Retinoids and phorbol esters alter release of fibronectin from enucleated cells  
(ornithine decarboxylase/retinoic acid)

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ABSTRACT Addition of 12-tetradecanoylphorbol 13-acetate (TPA) to cultures of intact Swiss mouse 3T3 fibroblasts induced a dose-dependent increase in ornithine decarboxylase (OrnDCase) activity. Over the same concentration range, \(10^{-9}\) to \(10^{-6}\) M, TPA induced the release of radioactively labeled fibronectin (FN) from the cells into the culture medium. Retinoic acid, a derivative of vitamin A, inhibited in a dose-dependent manner both the increase in OrnDCase activity and the release of FN induced by TPA. To examine the effects of TPA and retinoic acid in enucleated cells, the cells were treated with 7.5 \(\mu\)g of cytochalasin B per ml of medium during centrifugation at 10,000 \( \times \) g for 35 min at 37°C. In a series of five experiments, the treated cells were 94.7 \(\pm\) 4.8% (\(\pm\) SEM) enucleated as measured by \(^{3}H\)thymidine incorporation and verified by Giemsa staining for nuclei. In the enucleated cells, TPA did not induce increased OrnDCase activity but did induce FN release in a dose-dependent fashion over the same concentration range effective for FN release from intact cells. Moreover, addition of retinoic acid to the enucleated cells inhibited the phorbol ester-induced release of FN in a dose-dependent manner. A series of phorbol ester derivatives showed the same order of activity in causing FN release from the enucleated cells as reported for inducing OrnDase activity in intact cells or in promoting mouse skin tumors in vivo. Similarly, several retinoids were tested for their ability to inhibit the phorbol ester-induced release of FN from enucleated cells. The efficacy of the retinoids in preventing FN release paralleled their activity in inhibiting phorbol ester-induced OrnDCase activity and skin tumor promotion, as reported in the literature. We conclude that at least one aspect of tumor promotion induced by phorbol esters—the loss of FN—does not require the cell nucleus, and further, that retinoids can inhibit this aspect of tumor promotion without nuclear involvement.

From the recent work of Liu et al. demonstrating the binding of retinol to chromatin (1) and that of Fuchs and Green on the regulation of specific messenger RNA by retinol (2), one can safely conclude that in its normal physiological function, vitamin A can exert its action through the nucleus. Conversely, ample evidence supports an action of retinol and other retinoids on the cell surface membrane, especially by influencing membrane glycoproteins (3, 4). However, the cell surface action could still be mediated through an influence on protein synthesis via the nucleus. Therefore, we were interested in determining whether one of the cell surface actions of retinoids could be exerted in cells deprived of their nuclei. Tumor promoters elicit a range of biochemical responses in cells in culture, many of which mimic the activities of transformed or cancer cells. Among these are: stimulation of deoxyglucose uptake (5), induction of ornithine decarboxylase (OrnDCase) activity (6, 7), decreased cell-substratum adhesion (8–10), anchorage-independent growth (10, 11), changes in cell surface glycoproteins (10, 12), and loss of cell surface fibronectin (FN) (13, 14). In addition 12-tetradecanoylphorbol 13-acetate (TPA), a potent tumor promoter, can induce morphological changes in enucleated mouse skin fibroblasts (15).

Retinoids, and particularly retinoic acid (RA), inhibit many of these TPA-induced effects, including OrnDCase induction (16–18), decreased cell adhesion, and alteration of cell surface glycoproteins (10). Addition of RA to transformed cells increases their adhesion to substratum (19) and addition to normal cells increases incorporation of radioactive precursors in a M, 230,000 glycoprotein (20). Addition of RA to cultures of chondrocytes causes them to accumulate FN as a major cell surface glycoprotein (21). Aware that RA inhibits many of the actions of TPA and that TPA can induce morphological changes in cells without nuclei, we asked the following questions: Does TPA induce FN loss in Swiss mouse 3T3 cells? Can RA inhibit the TPA-induced loss of FN? Do enucleated 3T3 cells lose FN when treated with TPA? Can RA prevent the TPA-induced loss of FN in enucleated cells?

MATERIALS AND METHODS

Materials and their sources were: Swiss mouse 3T3 cells generously provided by Howard Green (Massachusetts Institute of Technology); Dulbecco's modified Eagle's medium (DME medium) and calf serum (Flow Laboratories); tissue culture dishes and flasks (Falcon); cytochalasin B (CB), crosslinked bovine serum albumin (Sigma); phorbol esters (P-L. Biochemicals); all-trans RA (Kodak); 13-cis RA (BASF); phenyl and furyl RA derivatives (Hoffman-LaRoche); IgG Sorb (Enzyme Center, Cambridge, MA); goat anti-rabbit IgG immunobeads (Miles); \(\lambda\)-[1-\(^{14}\)C]ornithine (55.4 mCi/mmol; 1 Ci = 3.7 \(\times\) 10\(^{10}\) becquerels) and [6-\(^{3}\)H]thymidine (16.2 Ci/mmol) (New England Nuclear); \(\lambda\)-[2,3,4,5-\(^{3}\)H]leucine (120 Ci/mmol) (ICN).

Cell Cultures. Swiss mouse 3T3 cells were grown in DME medium supplemented with 10% calf serum, 100 units of penicillin G and 100 \(\mu\)g of streptomycin per ml. Cells were passaged every 3–4 days and were used before the 10th passage. On the day before cells were to be enucleated, they were harvested with 0.1% trypsin and plated at 6 \(\times\) 10\(^{4}\) cells per 25-cm\(^{2}\) tissue culture flask to give a confluent layer of cells 18 hr later. All cultures were kept in 10% CO\(_{2}\)/90% air.

Enucleation. Cells were enucleated by the method of Nagle and Blumberg (15), except that the tissue culture flasks were not coated with collagen. Tissue culture flasks (25 cm\(^{2}\)) containing a confluent layer of cells were filled to the neck with

Abbreviations: TPA, 12-tetradecanoylphorbol 13-acetate; OrnDCase, ornithine decarboxylase; DME medium, Dulbecco's modified Eagle's medium; PhMeSO\(_{2}\)F, phenylmethanesulfonyl fluoride; CB, cytochalasin B; FN, fibronectin; RA, retinoic acid.

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DME medium supplemented with 10% calf serum and 7.5 μg of CB per ml. The flasks were placed in the GSA rotor of a Sorvall centrifuge, the chambers in the rotor were filled to the shoulder of the flasks with 37°C water, and the flasks were centrifuged at 10,000 × g for 35 min. The cells were washed three times with Ca²⁺- and Mg²⁺-free Dulbecco’s phosphate-buffered saline. The extent of enucleation was determined by Giemsa staining for nuclei and by [³H]thymidine incorporation into tri-chloroacetic acid insoluble material. After enucleation, fresh medium was added and 3 hr later the cells were exposed to a 30-min pulse of 40 μCi of [³H]thymidine. In a series of five experiments, the cells were shown to be 94.7 ± 4.8% (± SEM) enucleated as measured by thymidine incorporation.

Treatment with Phorbol Esters and Retinoids. Phorbol esters and retinoids were dissolved in ethanol and added to the medium of intact or enucleated cells to give a concentration of ethanol not greater than 0.1%.

Radioactive Labeling of Cells for Determination of FN Release. To determine FN release from the cells, the cells were labeled by a 4-hr incubation in 2.0 ml of leucine-free DME medium containing 10% calf serum and 70 μCi of [³H]leucine. After incubation, the cells were washed twice with DME medium. In enucleation experiments, the cells were enucleated at this point. Enucleated or intact cells were then incubated with DME medium without serum, with phorbol esters or retinoids (or both). Control cultures received neither phorbol esters nor retinoids. At the end of incubation, media were collected and cells were extracted with 4 M urea and 0.5% deoxycholic acid. Incorporation into tri-chloroacetic acid-precipitable protein was determined. Protein was measured by the method of Lowry et al. (22).

 OrnDCase Determination. Intact or enucleated cells were incubated for 4 hr in DME medium containing 10% calf serum with phorbol esters or retinoids (or both). Cells were collected by scraping and were sonicated twice for 15 sec in 1 ml of homogenization buffer (50 mM phosphate buffer, pH 7.2/0.1 mM EDTA/0.1 mM pyridoxal 5-phosphate). The supernatant was collected by centrifugation at 10,000 × g for 30 min at 4°C. OrnDCase (l-ornithine decarboxylase, EC 4.1.1.17) activity in the supernatant was determined by a modification of the microassay method of LaPointe and Cohen (23). Supernatant (50-100 μl) was incubated with 200 μl of incubation buffer (0.1 M phosphate buffer, pH 7.2/0.1 mM pyridoxal 5-phosphate/1 mM dithiothreitol/0.2 mM l-ornithine) and 0.02 μCi of l-14C]ornithine. After 30, 60, or 120 min, the reaction was stopped by addition of 50 μl of 2 M citric acid and the samples were incubated for another 15-20 min. The filter discs were removed and placed in scintillation vials containing 10 ml of a toluene-based scintillation fluid with Triton X-100, and the radioactivity was counted. OrnDCase activity is expressed as pmol of 14CO2 released per hr per mg of protein.

FN Immunoprecipitation. Mouse FN was isolated from mouse plasma by affinity chromatography on gelatin-Sepharose 4B. Unbound protein was eluted with 0.1 M phosphate buffer (pH 7.4) containing 0.1 M sodium citrate and 0.15 M NaCl. The retained protein was eluted with 4 M urea. The FN purity was determined by NaDodSO4/polyacrylamide gel electrophoresis to be >95%. Rabbit anti-mouse FN antibody was prepared by multiple injections of purified FN into a female New Zealand White rabbit. The rabbit anti-mouse FN antibody was purified by affinity chromatography on mouse FN-Sepharose 4B. Bound antibody was eluted with 0.1 M glycine/HCl buffer, pH 2.8, and was shown to be specific for FN by double immunodiffusion by using the method of Ouchterlony (24).

Immunoprecipitation was performed as follows: triplicate 50- to 300-μl samples in buffer (50 mM Tris-HCl, pH 8.3/50 mM NaCl/0.5% deoxycholic acid/2 mM phenylmethylsulfonyl fluoride (PhMeSO2F) to make 400 μM) were incubated with 10 or 20 μl of purified anti-mouse FN antibody for 1 hr at 37°C. Three hundred microliters of a 10% slurry of IgG sorb or 300 μl of goat anti-rabbit IgG immunobeads were added and the tube was incubated for another hour at 37°C (IgG sorb) or overnight at 4°C (immunobeads). The tube was centrifuged in a Beckman Microfuge at 12,000 × g for 4 min. The pellet was washed three or four times and transferred to a scintillation vial, and the radioactivity was counted. Incubation mixtures containing sample, but no antisera, were precipitated to measure nonspecific precipitation. Preimmune serum was substituted for immune serum as a control and did not precipitate radioactivity different from the nonspecific background, routinely <10% of the added radioactivity. For each sample the nonspecific radioactivity was subtracted from the total precipitated radioactivity to give specifically bound radioactivity. The immunoprecipitates were analyzed by NaDodSO4/polyacrylamide gel electrophoresis. In all cases the radioactivity that was precipitated by the immune serum migrated with authentic mouse FN; preimmune serum precipitates gave no bands. Ten microliters of immune serum is sufficient to precipitate 10 μg of FN in this assay, an amount in excess of the FN (15) released by TPA treatment from chicken embryo or human lung fibroblasts (14).

NaDodSO4/polyacrylamide gel electrophoresis was performed in 0.8- or 1.5-mm slab gels with 5% separating gels and 3% stacking gels with the buffers of Weber and Osborn (25). The gels were stained for protein with Coomassie blue G or subjected to fluorography after being soaked in ENHANCE. Crosslinked bovine serum albumin and mouse or human FN were used as molecular weight markers.

RESULTS

OrnDCase Induction and FN Release by TPA in Intact Cells. TPA induced dose-dependent OrnDCase activity between 10^-9 and 10^-6 M (Fig. 1). These TPA-induced cells showed a peak of OrnDCase activity between 4 and 5 hr, al-
though the activity was detected as early as 2 hr and continued for \( \approx 8 \) hr. TPA caused a dose-dependent increase in FN in the medium of cells treated with the same concentrations of TPA that were effective in OrnDCase induction (Fig. 1). TPA-induced release of FN was detected as early as 1 hr after TPA treatment and continued to increase with longer treatment times, up to 24 hr, the longest time tested. The FN precipitated by the anti-FN antibody migrated to the same position as authentic intact mouse FN after NaDodSO\(_4\)/polyacrylamide gel electrophoresis and did not appear to be a product of proteolytic cleavage. Similar results were reported with TPA-induced FN release from human lung fibroblasts (14).

**Inhibition of TPA-Induced OrnDCase and FN Release by RA.** Fig. 2 shows the dose-dependent inhibition of TPA-induced OrnDCase activity by RA. In cultures treated with \( 10^{-7} \) M TPA, RA effectively inhibited OrnDCase activity at \( 10^{-8} \) M, and at \( 10^{-6} \) M it reduced the OrnDCase activity to that of cells without TPA treatment. Over the same concentration range, RA inhibited the TPA-induced release of FN, such that at \( 10^{-6} \) M RA, the TPA-treated cell cultures were releasing an amount of FN nearly equal to that of untreated cells. Having established that TPA induced FN release and that RA inhibited this action of TPA, we examined the effect of TPA and RA on enucleated cells.

**Induction of OrnDCase Activity in Enucleated Cells.** We investigated the induction of OrnDCase activity in intact cells treated with CB, with and without centrifugation. CB was used to disrupt the cell cytoskeleton to allow enucleation. Half of the cultures of intact cells were treated with CB for 30 min without centrifugation. These cells retained their nuclei and appeared to have normal morphology after a subsequent 4-hr incubation in fresh DME. To prevent enucleated cells, cultures were treated for 30 min with CB during centrifugation. The enucleated cells incorporated 96.4% less thymidine than did the intact CB-treated cells without centrifugation. Giemsa staining for nuclei showed that nearly all of the cells subjected to enucleation lacked nuclei but were still attached to the culture dishes. After treatment with CB, with and without centrifugation, the media were changed and \( 10^{-7} \) M TPA or \( 10^{-7} \) M RA, or both, were added to the cultures; control cultures received no additions (Fig. 3). RA treatment of intact cells inhibited TPA-induced OrnDCase activity \( \approx 50\% \). In cells receiving RA alone, the OrnDCase activity was slightly less than that in control cells. In the enucleated cells receiving no additions, TPA and RA, or RA alone, there was no detectable OrnDCase activity. TPA-treated enucleated cells had \( \approx 3\% \) of the OrnDCase activity found in TPA-treated intact cells. This residual OrnDCase activity might have been the product of enzyme induction in enucleated cells or, more likely, was normal induction of OrnDCase in the 3–4% of the cells in enucleated cultures that retained their nuclei. For the most part, enucleated cells were incapable of responding to TPA treatment with OrnDCase induction.

The level of OrnDCase in intact cells not treated with CB (Figs. 1 and 2) was lower than that of intact CB-treated cells (Fig. 3). Apparently CB itself caused increased basal OrnDCase activity.

**Release of FN from Enucleated Cells.** Fig. 4 summarizes the analyses of FN in the media of intact and enucleated cells treated with TPA or RA, or both. To enucleate the cells, the cultures were exposed to CB, with and without centrifugation. In the absence of CB, centrifugation had no effect on FN release (data not shown). CB, without centrifugation, caused no increase in FN release from control cells during the 4-hr incubation after the removal of CB. However, in CB-treated cultures, TPA caused an increase in FN release compared with FN release from cells treated with TPA without prior exposure to CB. It appears that CB rendered the cells more susceptible to TPA-induced loss of FN.

In enucleated cells TPA caused a 76% increase in FN release compared with control cells, whereas in intact cells TPA caused a 62.5% increase in FN release compared with control.

![Fig. 2](image)

**Fig. 2.** RA inhibition of TPA induction of OrnDCase activity and FN release. To determine OrnDCase activity (○), cells were incubated with \( 10^{-7} \) M TPA plus various concentrations of RA for 4 hr. Each point represents the mean \( \pm \) SEM of two determinations carried out on duplicate dishes of cells. To determine FN in the medium (●), \( ^3\)Hlucine-labeled cells were incubated in fresh medium containing \( 10^{-7} \) M TPA plus various RA concentrations. After a 4-hr incubation, the media were assayed for FN by immunoprecipitation. Each point represents the mean \( \pm \) SEM of three determinations on duplicate dishes of cells. ○, FN in the medium of cells treated with RA and no TPA; OrnDCase activity and FN in the medium are expressed as in Fig. 1.

![Fig. 3](image)

**Fig. 3.** OrnDCase activity in intact and enucleated cells. Confluent layers of cells were incubated in DME medium containing 10% calf serum and 7.5 \( \mu \)g of CB per ml. Half of the flasks were centrifuged at 10,000 \( \times \) g at 37°C for 30 min to enucleate the cells; the remaining flasks were incubated without centrifugation for 30 min at 37°C. The cells were washed, fresh DME medium was added containing no additions (control), \( 10^{-7} \) M TPA, \( 10^{-7} \) M RA, or \( 10^{-7} \) M TPA and \( 10^{-7} \) M RA, and the cells were incubated for 4 hr. Each point represents the mean \( \pm \) SEM of two determinations of duplicate flasks of cells. A + sign indicates CB treatment with centrifugation; a − sign indicates CB treatment without centrifugation. OrnDCase activity is expressed as in Fig. 1.
though enucleation itself raises the basal level of FN release, TPA causes a similar percent increase in FN release in enucleated and intact cells.

Addition of $10^{-7}$ M RA with TPA to enucleated cells inhibited FN release by 33%, whereas in intact cells RA inhibited TPA-induced FN release by 40%. In enucleated cells treated with RA alone, very little FN release occurred.

**Effect of TPA Analogs on FN Release in Enucleated Cells.**

Driediger and Blumberg have demonstrated that in chicken embryo fibroblasts the ability of various phorbol esters to induce deoxyglucose uptake correlates well with their activity in inducing the loss of cell surface FN (5). The same phorbol ester structure–activity relationship was demonstrated in induction of morphological change in intact or enucleated Swiss mouse 3T3 cells (15) and in the induction of skin tumors (26). Similarly, we examined the efficacy of four phorbol esters in inducing FN release from enucleated cells (Fig. 5). TPA, the most potent tumor-promoting ester, was the most active phorbol ester in inducing FN release. 20-Oxo-20-deoxyphorbol 12-myrystate 13-acetate is active in tumor promotion and was slightly less effective in inducing FN release. Phorbol 12,13-dibenzate, a moderate tumor promoter, was moderately active in inducing release of FN. Finally, 4-O-methyl-phorbol 12-myrystate 13-acetate, a very weak promoter, was a very poor inducer of FN release. The activities of these phorbol esters in inducing FN release paralleled their activities in OrnDCase induction in intact cells (data not shown).

**Effect of Retinoid Analogs on TPA-Induced FN Release in Enucleated Cells.**

We tested a series of retinoids for their ability to inhibit FN release in enucleated cells treated with $10^{-7}$ M TPA (Fig. 6). The structure–activity relationship for retinoids has been well established in two systems: inhibition of skin tumor promotion by TPA (16) and reversal of keratinization in hamster tracheal organ culture (27). We compared the biological activity of retinoids reported in these systems with the efficacy of five retinoids in inhibiting TPA-induced FN release in enucleated cells. All-trans RA, the most potent inhibitor of skin tumor promotion, was the most active retinoid in inhibiting FN release. 13-Cis RA, only slightly less active than all-trans RA in tumor inhibition, was slightly less active than all-trans RA in inhibition of FN release. Retinyl acetate, which is of intermediate biological activity in inhibiting tumor promotion, was of intermediate activity in preventing FN release. The phenyl derivative of RA is a weak effector of keratinization and was weakly active in inhibiting FN release. Finally, the furyl derivative of RA, a very poor keratinization reverser, was only weakly active in inhibiting FN release. Similarly, we found that the activity of the retinoids in inhibiting TPA-induced loss of

**FIG. 4.** FN release from intact and enucleated cells. [$^3$H]leucine-labeled cells were treated with media containing 10% calf serum with no additions (open bars) or with CB at 7.5 µg/ml (filled and striped bars). Half of the CB-treated flasks were centrifuged at 10,000 × g for 30 min to enucleate the cells. The remaining flasks were incubated for 30 min at 37°C. The cells were then washed and fresh DME medium containing no additions (control), $10^{-7}$ M TPA, $10^{-7}$ M RA, or $10^{-7}$ M TPA and $10^{-7}$ M RA was added and the cultures were incubated for 4 hr. The media were assayed for FN by immunoprecipitation. Each point represents the mean ± SEM of three determinations on duplicate flasks of cells. (¢), TPA; (O), 20-oxo-20-deoxyphorbol 12-myrystate 13-acetate; (λ), phorbol 12,13-dibenzate; (A), 4-O-methyl-phorbol 12-myrystate 13-acetate. FN in the medium is expressed as in Fig. 1.

**FIG. 5.** Dose–response curves for FN release by phorbol ester derivatives. [$^3$H]leucine-labeled cells were enucleated by treatment with CB and centrifugation. Fresh DME medium was added containing various concentrations of phorbol ester derivatives. After a 4-hr incubation, the media were harvested and assayed for FN by immunoprecipitation. Each point represents the mean ± SEM of three determinations carried out on duplicate flasks of cells. (¢), TPA; (O), 20-oxo-20-deoxyphorbol 12-myrystate 13-acetate; (λ), phorbol 12,13-dibenzate; (A), 4-O-methyl-phorbol 12-myrystate 13-acetate. FN in the medium is expressed as in Fig. 1.

**FIG. 6.** Dose–response curves for inhibition of TPA-induced release of FN by retinoids. [$^3$H]leucine-labeled cells were enucleated by treatment with CB and centrifugation. Fresh DME medium was added containing $10^{-7}$ M TPA and various concentrations of retinoids. After a 4-hr incubation, the media were harvested and assayed for FN by immunoprecipitation. Each point represents the mean ± SEM of three determinations on duplicate flasks of cells. (¢), All-trans RA; (O), 13-cis RA; (λ), retinyl acetate; (A), 2-furyl analog of RA. FN in the medium is expressed as in Fig. 1.
FN correlated with their activity in inhibition induction of OrnDCase activity in intact cells (data not shown).

**DISCUSSION**

The tumor promoter, TPA, induces normal cells to assume many of the phenotypic characteristics of transformed cells. In this paper we have extended the findings of Keski-Oja et al. and Driedger and co-workers, who reported that TPA induces FN loss from human lung fibroblasts (14) and chicken embryo fibroblasts (13), to show that TPA also induces FN loss from mouse skin fibroblasts. Nagle and Blumberg have reported that these same 3T3 cells respond to TPA treatment with increased deoxyglucose uptake and altered morphology and have demonstrated a TPA-induced alteration in morphology in enucleated cells (15). We have now shown that TPA can induce FN loss in enucleated cells and, further, that addition of RA to TPA-treated cells inhibits FN loss.

It has been reported that CB induces the loss of cell surface FN (28). In these studies, the cells or media or both were examined immediately after the cells were treated with CB. In our experiments, we examined FN release into cell culture medium 4 hr after the removal of CB. We cannot say that CB does not induce some FN release in the 30-min treatment during enucleation, but we do report that after the subsequent 4-hr incubation without CB there is no difference between the level of FN release by control cells with or without prior exposure to CB. It has been reported that cells contain their normal complement of FN within 1 to 2 hr after CB has been removed (29). We believe, and microscopic examination of the cells shows, that after 4 hr of incubation the cells previously exposed to CB have a normal morphology, consistent with their having a normal complement of FN.

Although CB does not effect FN release from control cells during the 4-hr incubation, it does increase the sensitivity of the cells to TPA treatment. We suggest that the disruption of the cytoskeleton caused by CB or enucleation changes the attachment of FN to the cell and renders the cells more susceptible to TPA-induced release of FN. Conversely, we find that RA treatment without TPA results in a very small FN release, suggesting that RA-induced accumulation of FN may be enhanced in enucleated cells.

Of particular interest is the finding that in enucleated cells RA inhibits the TPA-induced loss of FN. Although the findings of Liu et al. demonstrate that retinol can reach and interact with nuclear components via transport with retinol-binding protein (1), we believe that the results given in this report argue for a nonnuclear involvement for retinoids, at least in their action as antitumor promoting agents.

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