Acquisition of a Growth-inhibitory Response to Phorbol Ester Involves DNA Damage

Yi Sun, Yves Pommier, and Nancy H. Colburn

ABSTRACT

TPA (12-0-tetradecanoylphorbol-13-acetate), a potent tumor promoter, has been shown to stimulate or inhibit cell growth depending on the cell type investigated. We recently found that RT101 cells, a transformed mouse JB6 epidermal cell line, acquired a greater growth inhibition response to TPA during conventional subcultivation. The growth of low-passage RT101 cells was slightly inhibited by TPA in monolayer culture but stimulated in soft agar. In contrast, the growth of high-passage cells was greatly inhibited by TPA in both monolayer culture and in soft agar. Inhibition was dose dependent, directly correlated with protein kinase C-activating activities of tumor promoters, and was found to be reversible. TPA-treated high-passage cells were greatly reduced in volume, showed extensive abnormal mitoses, and were more susceptible to detachment. High-passage cells were also found to be less tumorigenic as indicated by in vivo tumorigenicity assays in nude mice. TPA treatment rendered less tumorigenic in the case of both cell lines. The mechanism for acquisition of increased sensitivity to TPA of RT101 cells during subculture was investigated; it involved nondual DNA damage and detachment of nonviable cells. The results suggest the possibility that early-passage RT101 cells contained two subpopulations, one TPA-sensitive and one TPA-resistant population. Conventional subcultivation may have selected for the former subpopulation. The sensitive subpopulation may have been irreversibly inhibited as a result of TPA-induced cell killing, possibly apoptosis.

INTRODUCTION

Growth inhibition can occur irreversibly or reversibly. Irreversible growth inhibition has been attributed to four major mechanisms: (a) terminal differentiation; (b) apoptosis (programmed cell death); (c) cellular senescence; and (d) necrosis. Reversible growth arrest, however, can be induced by extrinsic factors, such as soluble growth-inhibitory substances, or by intrinsic mechanisms involving blocks in signal transduction or cell cycle progression (1).

Among irreversible growth inhibition processes, terminal differentiation has been well studied. TPA, a potent tumor promoter, induces terminal differentiation in a variety of cell types (2-4). Tumor cells originating from rodents and humans were found to be more resistant to TPA-induced terminal differentiation than their normal cell counterparts (5-9). Opposite TPA responses have been reported in heterogeneous subpopulations of mouse epidermal basal cells. TPA induced one subpopulation to differentiate, while another was stimulated to proliferate (10). It was proposed that resistance to TPA-induced terminal differentiation is an essential change in initiated cells in skin and is characteristic of malignant epidermal cells (5, 6, 11).

Apoptosis or programmed cell death has been observed in cells of different origins under physiological conditions (12) as well as after a number of treatments including glucocorticoids (13, 14), calcium ionophore (15, 16), and some chemotherapeutic drugs (17, 18). This form of cell death is characterized by cell membrane blebbing, cytoplasmic shrinkage, condensation of nuclear chromatin, and fragmentation of DNA. Endonuclease and drug-induced topoisomerase II inhibition were found to play roles in this process (12, 17).

We report here that a tumorigenic cell line, RT101, derived from mouse epidermal JB6 cells acquired an increased sensitivity to TPA-induced cytotoxicity during conventional subcultivation. The acquired sensitivity response to TPA shows properties reminiscent of apoptosis.

MATERIALS AND METHODS

Materials

Bovine serum albumin, nitroblue tetrazolium, xanthine, xanthine oxidase, glutathione (reduced form), glutathione reductase, sodium azide, β-NADPH, imidazole, horse radish peroxidase, diaminodisulfonic, formamide, 3-(morpholino)propanesulfonic acid, and superoxide dismutase-polyethylene glycol were purchased from Sigma (St. Louis, MO). Bathocuproine disulfonic acid, disodium salt hydrate, diethylenetriaminepentaacetic acid, and benzoyl peroxide were the products of Aldrich (Milwaukee, WI). Sodium cyanide, hydrogen peroxide, EDTA, phenol reagent solution, and formaldehyde were obtained from Fisher (Springfield, NJ). Bovine liver Cu,ZnSOD was from Diagnostic Data (Mountain View, CA). TPA, phorbol diacetate, and phorbol dibutyrate were from Chemicals for Cancer Research, Inc. (Eden Prairie, MN). DMSO was from Pierce (Rockford, IL) and Minimum essential medium Eagle was from Whittaker Bioproducts, Inc. (Waldersville, MD). Agarose was from BRL (Gaithersburg, MD). Zatabind membrane was from Cuno, Inc. (Meriden, CT). RNAzol solution was from Cinna/Biotex (Friendswood, TX). [3H]Thymidine was from NEN (Boston, MA). Trypan blue stain (0.4%) was from Gibco (Grand Island, NY). EGF was from Collaborative Research, Inc. (Bedford, MA), and telocidin was a gift from Dr. H. Fujiki (Tokyo).

Probes

c-jun was a gift from Dr. M. Birrer (19), and jun-B and jun-D were gifts from Dr. D. Nathans (20, 21). v- fos was a gift from Dr. T. Curran (22), P80 complementary DNA was a gift from Dr. S. Simek (23), and β-actin was from Dr. D. Cleveland (24).

Cell Cultures

The transformed cell line RT101 was derived originally from a primary mouse epidermal culture (25, 26). Cells at passages below and above 200 were referred to as low and high passage, respectively, since the change of transformation phenotype in soft agar after TPA treat-
ment occurred around passage 200. The cells with passages between 80 and 120 (low passage) and between 260 and 300 (high passage) were used for all experiments except for soft agar assays, in which cells at various passages were used. Cells were grown in Minimum essential medium Eagle containing 5% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO₂ in air. The effect of phorbol esters on monolayer growth of both high- and low-passage RT101 cells was measured in a one-step growth assay. Two-mL cultures were started at low cell density (1 x 10⁵ cells) in 12-well plates with different concentrations of phorbol esters, EGF, telocidin, benzoyl peroxide, or TPA plus SOD and cultured for 5 days without refeeding. Cells were counted every day, and the exponential growth phase was used for the calculation of growth rate. To test the reversibility of the TPA effect, the medium containing various concentrations of TPA was aspirated at day 5, and monolayers were rinsed twice with ice-cold PBS and refed with fresh medium without TPA. Cell viability was measured by trypan blue exclusion (27).

TPA Treatments

Cells for antioxidant enzyme assays and Northern analysis were treated as follows. TPA in DMSO was diluted (1:100) with Minimum essential medium Eagle and added directly to late exponential cells without medium change at a final concentration of 10 ng/mL. After the indicated incubation time, monolayers were rinsed twice with ice-cold PBS and either scraped with a sterile rubber policeman (for enzyme assay) or trypsinized (for Northern analysis). The cells were pelleted for 3 min at 2000 x g and stored at -70°C until assayed.

Soft Agar Assays

Ten thousand single cells were suspended in 0.33% agar containing 10% fetal bovine serum in a 60-mm dish in the presence or absence of TPA (16 ng/mL). Colonies of eight or more cells were counted after 14 days (25).

Nude Mice Tumorigenicity

Cells at the late exponential phase were treated with TPA (10 ng/mL) for 5 days and 14 days, respectively, with a medium change every 3 days. Two million attached viable cells suspended in PBS were injected s.c. into the upper back of a mouse (4 male and 4 female mice/group). Animals were checked weekly for signs of tumor growth.

Antioxidant Enzyme Activity Assays

Cell pellets were homogenized on ice in 50 mM potassium phosphate buffer, pH 7.8, with a Polytron (Brinkmann Instruments, Westbury, NY) using two 15-s bursts and sonicated with a Micro-Ultrasonic cell disrupter (Electro-Mechanical Instruments, Inc., Perkasie, PA) for 1 min in 15-s bursts, while on ice.

SOD Assays. Cu,ZnSOD and MnSOD activities were measured by the inhibition of nitroblue tetrazolium reduction as described by Sun et al. (28, 29). A standard inhibition curve using purified bovine liver SOD, EGF, telocidin, benzoyl peroxide, or TPA plus SOD and cultured for 5 days without refeeding. Cells were counted every day, and the exponential growth phase was used for the calculation of growth rate. To test the reversibility of the TPA effect, the medium containing various concentrations of TPA was aspirated at day 5, and monolayers were rinsed twice with ice-cold PBS and refed with fresh medium without TPA. Cell viability was measured by trypan blue exclusion (27).

Northern Blot Analysis

Total RNA was isolated from cells treated with TPA (10 ng/ml) using RNAzol solution according to the manufacturer's recommendations. Ten µg of total RNA were then size fragmented by electrophoresis on 1.2% agarose/formaldehyde gel, transferred onto Zetabind membrane, prehybridized, and hybridized according to the method of Church and Gilbert (35). The complementary DNA probes were labeled with random priming technique (36), to yield a specific activity of 2-5 X 10⁶ cpm/µg, and used at a concentration of 10⁶ cpm/ml hybridization solution. The final posthybridization wash was performed four times at 65°C for 10 min each time with a solution containing 1% SDS, 1 mM EDTA, and 20 mM sodium phosphate, pH 7.2. The membranes were then exposed to X-ray film (Kodak) with intensifying screens at −80°C. For the reprobing, the original probe was removed by treating the membranes with boiling water for 10 min.

RESULTS

A Stable Switch in the Response to TPA of JB6-derived Transformed RT101 Cells Occurred during Subcultivation. We have previously shown that the tumor promoter TPA can induce anchorage-independent growth in promotion-sensitive (P⁺) JB6 cells (25, 26). This transformation has been shown to be irreversible by the observation that cell lines cloned from agar colonies were tumorigenic and grew independently of anchorage in the absence of TPA. In some cells, such clonal lines showed enhanced growth in agar in response to TPA, but until now none has shown the inhibitory effects of TPA (25). As shown in Fig. 1, we found that TPA increased soft agar colony numbers in low-passage RT101 cells but greatly depressed (up to 5-fold) the ability of high-passage cells to grow in agar. In the absence of TPA, however, both cell lines have the ability to grow independently of anchorage, and the efficiency for the formation of soft agar colonies is similar (36% in high-passage versus 38% in low-passage cells). The results suggest that conventional subcultivation of RT101 cells accumulated some genetic or epigenetic changes in the cells, which led to an altered response to TPA treatment, while maintaining the transformed phenotype.

To address the question of whether TPA-induced inhibition of soft agar growth reflected a generalized growth-inhibitory effect, we performed a monolayer culture of RT101 cells in the presence of TPA or other phorbol esters. When growth in the
DNA DAMAGE AND GROWTH-INHIBITORY RESPONSE TO PHORBOL ESTER

Fig. 1. Depression of anchorage-independent growth by TPA in high-passage RT101 cells. Ten thousand single cells of low passage (passage 97-170) or high passage (passage 239-299) were suspended in 0.33% agar containing 10% fetal bovine serum in a 60-mm dish in the presence of TPA (16 ng/ml, 2.56 x 10^{-8} M) respectively. Control cells received 0.03% DMSO. Colonies of eight or more cells were counted after 14 days. Values are means ± SEM from five independent experiments.

0.03% DMSO solvent control was measured, no growth rate difference was found between high-passage and low-passage RT101 cells. They both doubled within 16 h (data not shown). As shown in Fig. 2 (top), when TPA, a very active tumor promoter, was included in culture, a concentration-dependent growth inhibition of monolayer RT101 cells was observed. The greater inhibition was seen in high-passage cells (29% of growth rate of untreated control cells versus 74% in low passage at a TPA concentration of 10 ng/ml, 1.6 x 10^{-8} M). Phorbol dibutyrate, a moderately active tumor promoter, also produced dose-dependent growth inhibition; again, high-passage cells were more sensitive. The magnitude of inhibition, however, was less than that observed with TPA, paralleling its transformation-promoting and relative PKC-activating activities (38). Phorbol diacetate, inactive as a tumor promoter, had no effect on the growth of either cell line. Furthermore, the monolayer growth inhibition caused by phorbol esters appeared to be specific to a protein kinase C signal transduction pathway, since telocidin, a protein kinase C activator with a transformation-promoting activity equal to that of TPA (data not shown), demonstrated a TPA-equivalent growth inhibition of RT101 cells, whereas epidermal growth factor (up to 20 ng/ml), a protein tyrosine kinase activator and transformation promoter (39, 40), had no inhibitory activity at all (Fig. 2, middle). Moreover, when benzoyl peroxide, a transformation promoter in JB6 cells not known to be a protein kinase C activator (41, 42), was tested, a similar dose-dependent growth inhibition was observed in both cell lines (Fig. 2, bottom). Finally, TNF-α, another transformation promoter not known to be PKC activator in JB6 cells (43), produced growth inhibition in RT101 with a preferential effect in low-passage cells (not shown). Taken together, the results indicated that monolayer growth inhibition, preferentially seen in high-passage cells, occurred not with tumor promoters in general but with PKC activators in particular.

Tumor Latent Period for RT101 Cells Was Lengthened by Passaging, and TPA Treatment Enhanced the Effect. The differential TPA responses of high-passage and low-passage RT101 cells in agar and monolayer have indicated that conventional subcultivation increased the sensitivity of cells to TPA-induced growth inhibition. To determine whether other aspects of the RT101 phenotype changed, we examined the tumorigenicity of both cell lines in nude mice. As illustrated in Fig. 3, untreated

Fig. 2. Monolayer growth inhibition of RT101 cells by phorbol esters. Ten thousand single suspended cells in 2 ml media were seeded in 12-well plates with different concentrations of phorbol esters, TN or EGF, or benzoyl peroxide, as indicated. Cells were cultured for 5 days without refeeding and were counted every day with a Coulter counter. The exponential growth phase was used for the calculation of growth rate. At least two independent experiments were conducted for each reagent, and values are the means from duplicate wells of a representative experiment. Tumor-promoting and PKC-activating activities follow the order TPA > phorbol dibutyrate > phorbol diacetate. H, high-passage RT101 cells (passage 285 for phorbol ester experiment, 295 for EGF, 305 for TN, and 271 for benzoyl peroxide); L, low-passage RT101 cells (passage 107 for phorbol ester, 117 for EGF, 127 for TN, and 109 for benzoyl peroxide); —, high-passage cells; — — —, low-passage cells. TPA: O, O, TPA; Δ, Δ, phorbol dibutyrate; ⊙, phorbol diacetate. MIDDLE: O, O, TN; Δ, Δ, EGF.
High-passage RT101 cells showed a mean latent period of 4.2 weeks, whereas low-passage cells showed a mean latent period of 1.5 weeks for tumor formation, indicating that high-passage cells are less tumorigenic. When cells were treated with TPA (10 ng/ml) for 5 or 14 days, we observed that the TPA treatment extended the mean latent period. The delay (relative to untreated cells) was 0 weeks in high-passage cells and 1.5 weeks in low-passage cells after 5 days of TPA treatment, and 0.5 and 1.5 weeks in high-passage and low-passage cells, respectively, after 14 days of treatment (Fig. 3). There is no indication that differential latent periods could be attributed to differential viability, since in all cases only attached cells were injected and they showed 95–100% viability at the time of injection. We conclude that compared to high-passage cells, the high-passage RT101 cells do not show enhanced sensitivity to a TPA-extended latent period but do show intrinsically lower tumorigenicity as manifested by a longer latent period for tumor formation in nude mice.

Differential jun and fos Basal Levels in High-Passage and Low-Passage RT101 Cells. We wished to understand the molecular mechanism of growth inhibition, observed preferentially in high-passage RT101 cells. When sensitive but not resistant EL4 mouse thymoma cells underwent phorbol ester-induced growth inhibition, the mechanism was shown to involve c-Jun and Fos-related protein induction (44). Since TPA is known to induce jun and other AP-1 gene expression in JB6 cells (45, 46), we asked whether the preferential growth inhibition in high-passage RT101 cells might involve jun or fos induction. Results showed that the induced levels of c-jun, jun B, and c-fos mRNA were similar in high-passage and low-passage RT101 cells (Fig. 4B and not shown), but basal levels of these mRNAs were lower in high-passage (3-fold for c-jun, 2-fold for jun B, and 5-fold for c-fos; Fig. 4A). TPA induced c-jun expression, with maximum induction seen at 2 h posttreatment. Although the fold induction at the peak was higher in high-passage cells, the actual amount of induced message was similar in the two cell lines. The c-jun message level decreased to basal levels by 6 h (Fig. 4B). The jun B and c-fos showed a similar TPA induction pattern after TPA treatment, with no significant difference detected between the two cell lines, while jun D was not induced (data not shown). As a negative control, expression of the mRNA for p80, a major phosphoprotein in JB6 mouse epidermal cells (23), was also measured at the mRNA level after TPA treatment, and no TPA response could be detected (not shown). The results indicated that the expression of nuclear oncogenes of the jun and fos families was rapidly but not differentially induced by TPA in the two cell lines. The quantitative differences seen in the basal levels of nuclear oncogenes between the two cell lines might, however, contribute to their differential latent period for tumor formation.

TPA Has No Significant Effect on Antioxidant Enzyme Activities in High-Passage and Low-Passage RT101 Cells. It has been shown that TPA acts as a strong tumor promoter through a mechanism that may be mediated by reactive oxygen (47, 48). TPA can produce oxygen radicals in polymorphonuclear leukocytes (49) and hydrogen peroxide in murine epidermal keratinocytes (50). Furthermore, TPA has been shown to inhibit antioxidant enzyme activities both in vivo and in vitro (48 and reviewed in Ref. 51). Antioxidant enzymes, the major cellular defense system against toxic oxygen radicals produced during normal metabolism and after oxidative insult, consist of Cu,ZnSOD, MnSOD, CAT, GPx, glutathione reductase, and glucose-6-phosphate dehydrogenase (51). We asked whether the TPA growth-inhibiting response preferentially seen in high-passage RT101 cells could be attributed to deficient cellular antioxidant enzyme protection. Three primary antioxidant enzyme activities were measured and shown in Fig. 5. Since morphological change could be observed 2 h after TPA treatment and relevant biological changes should occur prior to morphological change, two time points (2 h and 6 h) were
chosen. TPA has no significant effect on Cu,ZnSOD (Fig. 5, top), CAT (Fig. 5, bottom), and GPx activities (not shown). An inhibition of MnSOD activity was, however, found after TPA treatment with a 1.8-fold decrease at 6 h in high-passage cells, but no change was found in low-passage cells (Fig. 5, middle). Basal levels of Cu,ZnSOD and CAT were 1.6-fold and 1.8-fold higher in high-passage than in low-passage cells, respectively (Fig. 5, top and bottom). The basal level difference in antioxidant enzymes may not have a significant effect on the growth of the cells, since the doubling times for the two cell lines were found to be identical. The inhibition of MnSOD activity in high-passage cells, however, might be relevant to its greater sensitivity to TPA treatment.

Alterations of Cell Morphology by TPA in High-Passage and Low-Passage RT101 Cells. Accompanying the growth inhibition, cell morphology was dramatically changed (Fig. 6; compare C and D with A and B). Cell size was decreased due to cytoplasmic shrinkage, an effect that may result in less contact between cells. In fact, cell shrinkage can be observed 2 h after TPA treatment (not shown). While TPA produces cell shrinkage and reduced adhesiveness in both cases, only in the high-passage do we see extensive abnormal mitoses, broken cells, deficiency of normal mitosis, and a higher degree of detachment. The growth inhibition and morphological changes can, however, be reversed in the attached population after the removal of TPA. Fig. 6 (E and F) show cell morphology when TPA was removed at day 5 of treatment, and cells were rinsed twice with PBS and fed with fresh medium for 5 days. Although they were photographed at a higher density, they appeared very similar to each other and to Fig. 6 (A and B), showing a return to flatter, more adhesive cells with intact plasma membranes.

Cell Detachment Induced by TPA Involves DNA Damage. As mentioned earlier, TPA treatment caused cell detachment, especially in high-passage RT101 cells. To quantitate this TPA effect and to see whether detached cells were still viable, we performed a trypan blue exclusion experiment and found that the percentage detachment of high-passage cells increased as incubation time with TPA increased. At day 3, 60% cell detachment was observed, and the detachment level remained high (40%) afterward. Low-passage cells, however, had a very low percentage detachment (Fig. 7). No detachment was found in either cell line in the absence of TPA (not shown). Moreover, nearly all detached cells (95%) in both cell lines were stained by trypan blue and were identified as dead cells, and nearly all attached cells (95%) were viable and dividing cells. The data suggest that the apparent TPA-induced growth inhibition, especially that seen in high-passage cells, was due to its induction of cell detachment.

DNA damage after TPA treatment was measured in both attached and detached cells of both cell lines by alkaline elution assay (37). Little DNA damage in attached RT101 of either low-passage or high-passage cells was observed up to 72 h posttreatment (Figs. 8 and 9), and no significant difference could be seen between the two cell lines. As shown in Figs. 8 and 9, however, a high level of DNA damage in detached RT101 cells of both high and low passage was detected after TPA treatment. Large amounts of small DNA fragments were found in the lysis fractions of the detached cells (Fig. 8, time 0 of the elution curves). Since the pH of the lysis solution we used (pH 10) is too low to denature genomic DNA at room temperature, the DNA fragments found in the lysis fractions must be double-stranded. The elution curves of TPA-treated cells were not linear. Their slopes were steeper at early elution times, and after 3 h they became comparable to those of untreated cells (Fig. 8). The contrast between TPA-induced and γ-radiation-induced DNA damage indicates that in the case of TPA, DNA breaks were not randomly distributed (52). Further attempts to detect any difference in DNA damage in low-passage and high-passage TPA-treated RT101 cells analyzed 27,000 x g supernatants for characteristic nucleosome ladders (53). The results showed a similar pattern in high and low passage of 0.2–0.6, 0.75, and 1.5-kilobase DNA fragments separated by agarose gel electrophoresis (not shown). Taken together, these results are consistent with two cell populations existing in the detached cells for both cell lines such that approximately 90% of cells had their DNA completely fragmented and 10% of the cells had only minor fragmentation. Again in the case of detached

Fig. 5. TPA has no significant effect on antioxidant enzyme activities in high-passage and low-passage RT101 cells. Cells (high passage = 273–276; low passage = 95–98) at the late exponential phase were treated with TPA (10 ng/ml) for an indicated period of time. Cells were homogenized and sonicated, and antioxidant enzyme activities were measured as described in “Materials and Methods.” Top, Cu,ZnSOD; middle, MnSOD; bottom, catalase. The average enzyme activity values shown in the figures were obtained from four independent experiments on control cells and from three independent experiments on TPA-treated cells. Bars, SEM.

1911
DNA DAMAGE AND GROWTH-INHIBitory RESPONSE TO PHORBOL ESTER

Fig. 6. Reversibility of TPA-induced morphological changes in RT101 cells. Ten thousand cells were seeded in a 12-well plate and were grown for 5 days in the absence of TPA (A, low passage; B, high passage) and in the presence of 10 ng/ml of TPA (C, low passage; D, high passage). TPA induced obvious morphological changes. Cells were greatly shrunk and have decreased contact with each other. The morphological changes, however, were reversible. As shown in E (low passage) and F (high passage), cells were cultured with a fresh medium for 5 days after TPA was removed at day 5. Figure shows a representative area. X200.

cells, the difference of DNA damage in the two cell lines was not significant (Fig. 9). The results suggested that the differential TPA response seen in high-passage and low-passage RT101 cells was due to differential induction of cell detachment with associated DNA damage and cell killing.

DISCUSSION

The major observation described above is that mouse JB6-derived RT101 tumor cells underwent a stable change from relatively TPA insensitive to relatively TPA sensitive for growth inhibition. The acquired inhibitory response to TPA involved DNA damage and cell detachment and yet was reversible. Another phenotypic change which may or may not have been independent of the acquired sensitivity to TPA was the reduced tumorigenicity in high-passage cells. One interpretation of our observations is that there are two populations in RT101 cells, a TPA-sensitive subpopulation which was irreversibly inhibited by TPA and a TPA-resistant subpopulation, and that conventional subcultivation favored selection for the sensitive subpopulation. Alternatively, there may have been no preexisting sensitive subpopulation, but cells acquired a uniformly more TPA-sensitive phenotype due to genetic or epigenetic changes occurring during passaging. In this case, all cells in the population would have an equal probability of detaching, and the apparent resistance of the attached cells (high passage) might be attributable to physiological heterogeneity, such as being in the wrong phase of the cell cycle. The available data do not clearly distinguish between the two models. Nevertheless, perhaps the simplest explanation for the observed reversibility of growth inhibition upon removal of TPA and the lack of DNA damage in the attached cells is that at least a subpopulation is insensitive and this subpopulation is smaller at high passage.

As indicated earlier, induced growth inhibition might occur by a process of terminal differentiation, cellular senescence, necrosis, or apoptosis. Terminal differentiation appears unlikely because in TPA-induced terminally differentiated cells, DNA single-strand breaks were seen (52) instead of the double-strand breaks found with RT101 cells. Moreover, terminal differentiation-associated DNA damage occurred maximally at
DNA DAMAGE AND GROWTH-INHIBITORY RESPONSE TO PHORBOL ESTER

Fig. 7. TPA causes detachment of high-passage RT101 cells. Ten thousand cells in 2-ml culture were seeded in a 12-well plate in the presence of TPA (10 ng/ml) for an indicated period of time. Both detached (in the medium) and attached cells were harvested and stained with trypan blue (into a 450-μl cell suspension; 50 μl of 0.4% trypan blue was added). Cells were counted using a hemocytometer. Viable cells were scored for those having normal morphology and giving no appreciable staining with the dye. Percentage cell detachment was calculated by the ratio of cells in the medium over the total cells. As a control, 0.03% DMSO was used instead of TPA.

24 h instead of 6 h after TPA treatment (52). Cellular senescence is generally associated with limited lifespan cultures, not immortalized cell lines. Necrosis also appears unlikely, since it shows distinct morphological characteristics of swollen cells and damaged membranes. Furthermore, DNA damage found in necrosis consists of indiscriminate nicks and breaks and occurs slowly (54). The existing data are consistent with a mechanism involving TPA-induced apoptosis characterized by cytoplasmic shrinkage at 2 h after TPA treatment, nonviability of detached cells, and nonrandom DNA fragmentation by 6 h.

Little is known about the signal transduction pathway(s) that trigger processes of programmed cell death such as apoptosis. In the RT101 cells, TPA-induced putative apoptosis appears to be mediated by a protein kinase C pathway, since the ability of phorbol esters to induce growth inhibition was correlated with their activity to activate protein kinase C. EGF, a protein tyrosine kinase but not a PKC activator, showed no inhibitory effect, although it has strong transformation-promoting activity. Benzoyl peroxide, another transformation promoter not known to be a PKC activator, produced a similar growth inhibition in the TPA-sensitive and TPA-resistant RT101 cells. In contrast, telocedrin, a PKC activating and transformation promoter, demonstrated a TPA-equivalent growth inhibition of RT101 cells, preferentially in high-passage cells (Fig. 2). There is evidence that PKC can modulate apoptosis in other cell systems. In cortical thymocytes, PKC activation by phorbol dibutyrate or TPA inhibits glucocorticoid-induced apoptosis (55), and in vascular endothelial cells in which apoptosis is induced by 2–4 h serum deprivation, TPA or fibroblast growth factor, which stimulates PKC, inhibits the apoptosis (56). In contrast, inhibition of C-kinase by H-7, but not of A-kinase by H-A, blocks hydrocortisone-induced apoptosis in mouse thymocyte (57). The modulating effects of PKC upon apoptosis are probably complex, since apoptosis appeared to be either induced (present study and Ref. 57) or suppressed (55, 56) by PKC activation.

Jun and/or fos induction has been shown to occur in JB6 cells undergoing a transformation response to TPA (45, 46) and in other cells during an antimitogenic response (58, 59) and a phorbol ester-induced growth inhibition (44). In both passage RT101 cells we found a similar magnitude of rapid induction of c-jun, jun B, and c-fos after TPA treatment, indicating that the high sensitivity of high-passage RT101 cells to TPA cannot be explained by greater jun or fos induction. The low basal

Fig. 8. Rapid induction of DNA damage by TPA in detached RT101 cells of either low or high passage. Cells were seeded at low density (10^5 cells/75-mm flask) and labeled with [3H]thymidine (0.03 μCi/ml) for 72 h. After the labeling medium was removed, TPA was added for 6 h. Attached and detached cells were harvested separately, and DNA damage was measured by alkaline elution as detailed in “Materials and Methods.”, L, untreated cells; H, cells treated with TPA for 6 h. 

In the RT101 cells, TPA-induced putative apoptosis appears to be mediated by a protein kinase C pathway, since the ability of phorbol esters to induce growth inhibition was correlated with their activity to activate protein kinase C. EGF, a protein tyrosine kinase but not a PKC activator, showed no inhibitory effect, although it has strong transformation-promoting activity. Benzoyl peroxide, another transformation promoter not known to be a PKC activator, produced a similar growth inhibition in the TPA-sensitive and TPA-resistant RT101 cells. In contrast, telocedrin, a PKC activating and transformation promoter, demonstrated a TPA-equivalent growth inhibition of RT101 cells, preferentially in high-passage cells (Fig. 2). There is evidence that PKC can modulate apoptosis in other cell systems. In cortical thymocytes, PKC activation by phorbol dibutyrate or TPA inhibits glucocorticoid-induced apoptosis (55), and in vascular endothelial cells in which apoptosis is induced by 2–4 h serum deprivation, TPA or fibroblast growth factor, which stimulates PKC, inhibits the apoptosis (56). In contrast, inhibition of C-kinase by H-7, but not of A-kinase by H-A, blocks hydrocortisone-induced apoptosis in mouse thymocyte (57). The modulating effects of PKC upon apoptosis are probably complex, since apoptosis appeared to be either induced (present study and Ref. 57) or suppressed (55, 56) by PKC activation.

Jun and/or fos induction has been shown to occur in JB6 cells undergoing a transformation response to TPA (45, 46) and in other cells during an antimitogenic response (58, 59) and a phorbol ester-induced growth inhibition (44). In both passage RT101 cells we found a similar magnitude of rapid induction of c-jun, jun B, and c-fos after TPA treatment, indicating that the high sensitivity of high-passage RT101 cells to TPA cannot be explained by greater jun or fos induction. The low basal
levels of jun and fos in high-passage cells may, however, contribute to their longer latent period for tumor formation.

TPA treatment does not produce substantial loss of antioxidant protection, implying that reactive oxygen may not be on the signal pathway for killing by TPA. In fact, exogenous addition of Cu,ZnSOD (5 µg/ml; 500 units/ml) only slightly (10%) reversed the growth inhibition induced by TPA (not shown). This marginal effect occurred even when superoxide dismutase was attached to polyethylene glycol to facilitate the uptake. It is noteworthy that high-passage RT101 cells were much more resistant than low-passage cells to growth inhibition induced by TNF-α, an elevator of cytotoxic levels of active oxygen in some cells (60) and a promoter of transformation in JB6 cells (43). This inhibitory effect of TNF-α on low-passage and high-passage RT101 cells was the opposite of that with TPA treatment. The higher levels of Cu,ZnSOD and catalase observed in the high-passage cells may play a protective role against TNF-α. The data suggest that growth arrest induced by TPA is mediated through a pathway different from that induced by TNF-α in RT101 cells. Moreover, higher levels of antioxidant enzymes in high-passage cells may also be relevant to their reduced tumorigenicity in nude mice (Fig. 3), since antioxidant enzyme activities have been reported to decrease with malignant progression (51).

It has been suggested that all cells undergo a similar program of apoptotic events, but different cells receive different signals that trigger apoptosis. Selective activation of endogenous endonuclease(s) has been postulated to play a major role in DNA damage occurring in apoptotic processes (12, 61, 62). DNA damage and cell detachment were prominent in TPA-induced putative apoptosis of RT101 cells. If this process is apoptosis, then nonrandom DNA damage is involved. The finding that the elution kinetics of TPA-treated RT101 cells were not first order indicates the presence of DNA breaks that were nonrandomly distributed among the cells analyzed on a single filter by alkaline elution (Fig. 8). It is noteworthy that benzoyl peroxide, which induces random DNA single-strand scission in a dose-dependent manner through a reactive oxygen pathway in JB6 cells (42), does not induce a preferential growth inhibition in high-passage cells (Fig. 2, bottom). At present, we cannot clearly distinguish which event occurs first in response to TPA. That is, does detachment occur as a consequence or as a cause of DNA damage? However, the observation that DNA damage was maximal (i.e., >90% of the detached cells) at 6 h but detachment was not maximal until 72 h (Figs. 7 and 9) suggests that DNA damage precedes and may be causally related to detachment. It will be of interest to examine the hypothesis that TPA via a PKC signal transduction pathway activates endonuclease(s), which cleaves DNA and cell detachment results. Moreover, apoptosis was recently reported to be blocked by Bel-2, an inner mitochondrial membrane protein (53), as well as by the products of Epstein-Barr virus latent genes (16). It will also be of interest to examine whether TPA-induced apoptosis can be blocked by the overexpression of bel-2 or Epstein-Barr virus genes.

ACKNOWLEDGMENTS

We wish to thank Edmund Wendel for carrying out the soft agar assays and D. Kerrigan for excellent technical assistance in performing the alkaline elution assays. We also thank Drs. A. Fornace and S. Yuspa for comments on the manuscript.

REFERENCES


DNA DAMAGE AND GROWTH-INHIBITORY RESPONSE TO PHORBOL ESTER


42. Gensler, H. L., and Bowden, G. T. Evidence suggesting a dissociation of DNA strand scissions and late-stage promotion of tumor cell phenotype. Carcinogenesis (Lond.), 4: 1507-1511, 1983.


Acquisition of a Growth-inhibitory Response to Phorbol Ester Involves DNA Damage

Yi Sun, Yves Pommier and Nancy H. Colburn


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/52/7/1907

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, contact the AACR Publications Department at permissions@aacr.org.