Promotion-sensitive epidermal and mammary epithelial cells maintained in suspension over agarose

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Abstract. The molecular basis of tumour promotion is still largely unknown. In *in vitro* model of tumour promotion, the promotion-sensitive cells are induced to grow under anchorage-independent conditions in the presence of promoting agent. The customary way of providing such conditions is to immobilize these cells in soft agar, but such cells cannot be readily recovered to study the induced biochemical and molecular events. In the present report, we analysed these events using JB6 mouse epidermal cells maintained in suspension in liquid medium over agarose. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) induced anchorage-independent synthesis of DNA in promotion-sensitive P+ (but not in promotion-resistant P–) JB6 cells and this TPA-induced synthesis of DNA positively correlated with TPA-induced formation of colonies in soft agar. The TPA-induced synthesis of DNA began on or shortly before 24 h after the introduction of TPA, peaked at about 48 h and then declined to the control levels over the next several days. All *trans*-retinoic acid and dexamethasone inhibited and calcitriol (1 α ,25-dihydroxy-vitamin D3) synergistically stimulated this TPA-induced DNA synthesis. Western immunoblot analysis of cyclins (A, B1, D1 and E) and p27Kip1, a cyclin-dependent kinase inhibitor, indicated that TPA induced cyclin A and cyclin B1 expression in P+ (but not in P–) JB6 cells and this induction coincided in time with TPA-induced synthesis of DNA. TPA also strongly induced cyclin D1 expression in P+ (but not in P–) JB6 cells, but this induction started prior to the expression of cyclin A and cyclin B1. TPA did not affect the expression of either cyclin E or p27Kip1 to any significant extent. We also found that NMU38 rat mammary epithelial cells were operationally equivalent to the promotion-sensitive P+ JB6 cells, but in these cells 17 β -oestradiol exerted a strong synergistic effect on TPA-induced synthesis of DNA. Based on these observations, we tentatively propose a sequence of molecular events which possibly lead to the anchorage-independent synthesis of DNA in these cells.

The process of carcinogenesis has been divided into three operationally distinct stages: initiation, promotion and progression (for a recent review article, see Sirica 1996). The

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mouse model of multistage skin carcinogenesis has for many years served as the experimental prototype for demonstrating such stages. The initiation stage reflects a permanent and irreversible change in the initiated cells, presumably due to mutational alterations in the cellular genome. The promotion stage is characterized by the selective proliferation of initiated cells resulting in their clonal expansion. The two most distinguishing characteristics of promotion are the operational reversibility and the fact that promoting agents exhibit a threshold effect. The progression stage represents the irreversible transition from benign (papilloma) to malignant (squamous cell carcinoma) lesions and finally to metastatic tumours.

Multistage carcinogenesis has been demonstrated in a variety of different tissues and organs of experimental rodent models including liver, lung, trachea, mammary gland, urinary bladder, prostate, thyroid, forestomach, pancreas and colon (Sirica 1996). Multistage theory of carcinogenesis has also been proposed for humans based on the epidemiological study on age distributions of age-specific incidence rates of common human cancers.

The initiated, promotion-sensitive JB6 mouse epidermal cells in culture have been serving as an *in vitro* paradigm to complement the *in vivo* model of the promotion phase of multistage mouse skin carcinogenesis (Colburn *et al.* 1978, 1979, Colburn, Koehler & Nelson 1980, Bernstein *et al.* 1991, Strickland *et al.* 1997). JB6 cells constitute a series of clonal genetic variants that include promotion-sensitive (P+), promotion-resistant (P-) and transformed (Tx) cells. In P+ cells, TPA induces the formation of large, tumorigenic, anchorage-independent colonies in soft agar at a high frequency. In contrast, P- cells exhibit a response in soft agar that is 0.1–1% that of P+ cells and the colonies are considerably smaller. Promotion of anchorage independence in P+ cells also occurs in response to many non-TPA promoters, making it a particularly useful system in identifying and elucidating different molecular mechanisms involved in tumour promotion (Colburn *et al.* 1980).

Since cells immobilized in soft agar cannot be readily recovered to study the induced biochemical and molecular events (Dong *et al.* 1994a), we analysed these events using JB6 cells maintained in suspension in liquid medium over agarose. We show here that TPA induces anchorage-independent synthesis of DNA in P+ (but not in P-) JB6 cells and that this TPA-induced synthesis of DNA positively correlates with TPA-induced formation of colonies in soft agar. We also show that NMU38 rat mammary epithelial cells are operationally equivalent to P+ JB6 cells, but in NMU38 cells 17β -oestradiol exerts a strong synergistic effect on TPA-induced synthesis of DNA.

MATERIALS AND METHODS

Cells

The promotion-sensitive (P+, clone 41.5a), resistant (P-, clone 30.7a) and transformed (Tx, clone R101) JB6 mouse epidermal cells were kindly provided by Dr N. H. Colburn (National Cancer Institute, Frederick, MD). The NMU38 rat mammary epithelial cells were provided *gratis* by Dr S. Sukumar (Johns Hopkins Oncology Center, Baltimore, MD). All cells were maintained in Eagle's Minimum Essential Medium (EMEM) containing 5% heat-inactivated fetal bovine serum (FBS), 2% L-glutamine, and antibiotics. They were passaged after trypsinization with 0.05% trypsin–0.02% EDTA solution. The cultures were always maintained below confluency. The cells were checked periodically for *Mycoplasma* by DNA fluorochrome staining (McGarrrity, Steiner and Vanaman 1983) and found to be contamination free.

Soft-agar assay

The JB6 cells (1×10^4) in log phase were suspended in 1.5 ml of 0.3% agar medium (top layer) over 7 ml of 0.5% agar medium (bottom layer) in a 60-mm dish (Dong *et al.* 1994a, Strickland *et al.* 1997). Each agar layer contained EMEM-NEAA (GIBCO BRL/Life Technologies, Grand Island, NY) supplemented with 10% FBS, 0.22% (wt/vol) sodium bicarbonate, and antibiotics. Either drug (in vehicle) or vehicle alone was applied in both layers. The agar dishes were incubated at 37°C for 14 days in a humidified atmosphere of 5% CO₂ in air. For determination of TPA-dependent colony induction, a dish containing cells in soft agar was placed over a template containing 1-cm² calibrations. Only clusters containing eight or more cells were scored as colonies. Colonies were counted in four 1-cm² areas randomly chosen with respect to distance from the centre of the dish, and the count was multiplied by an appropriate factor ($\times 5.5$) to give the colony number per 10 000 cells per 60-mm dish. All experiments were performed in triplicate.

Over-agarose suspension culture

The cells ($5\text{--}8 \times 10^5$) in log phase were suspended in 15 ml of liquid medium over 15 ml of 0.7% agarose medium (Ultra-Pure DNA Grade, Bio-Rad Laboratories, Richmond, CA) in a 100-mm dish. Each layer contained EMEM-NEAA supplemented with 10% FBS, 0.22% (wt/vol) sodium bicarbonate, and antibiotics. Either drug (in vehicle) or vehicle alone were applied in both layers. The dishes were incubated at 37°C for 0–6 days in a humidified atmosphere of 5% CO₂ in air.

Total RNA

The cells in the liquid layer (~ 15 ml) and three washings (3×5 ml culture medium) were collected in a 50-ml tube, centrifuged, and the pelleted cells were lysed in 1 ml of TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). Total RNA was isolated according to the manufacturer's protocol with a high-salt precipitation modification (Chomczynski & Mackey 1995).

Total protein

Total protein was isolated from the cells using the method of Wang, Posner & Hajimohammadreza (1996) with minor modifications. Briefly, the cells in the liquid layer and the three washings were collected in a 50-ml tube and centrifuged. The pelleted cells were suspended in 10 ml of Hank's balanced salt solution (HBSS). The tube was centrifuged again and the pelleted cells were resuspended in 500 μ l of HBSS. Fifty microlitres of 100 mM EDTA was added, followed by 500 μ l of lysis buffer (containing 2% (wt/vol) SDS, 5 mM EGTA, 5 mM EDTA, 0.5 mM PMSF, 10 μ g/ml AEBSF, 5 μ g/ml leupeptin, 10 μ g/ml pepstatin, 10 μ g/ml TLCK, 10 μ g/ml TPCK, 20 mM Tris-HCl (pH 7.4)). The cells were allowed to lyse for 10–15 min. The entire lysate was then transferred to a microcentrifuge tube and 100 μ l of 100% (wt/vol) trichloroacetic acid (TCA) was added to the lysate. After centrifugation, the pellet was washed with 1 ml of 2.5% (wt/vol) TCA. The final pellet was neutralized with 10 μ l of 3 M Tris base for 30–60 min and then diluted with 10 μ l of water. The solubilized protein sample (~ 20 μ l) was analysed for protein concentration using Bradford assay (Bio-Rad Laboratories, Richmond, CA). The sample was transferred to a fresh tube and stored at -20°C until use.

Protein immunoblotting

Whole cell protein (20 μ g) was separated on 7.5% or 12% discontinuous SDS-PAGE in a minigel unit, transferred to a Tropifluor PVDF membrane (Tropix, Bedford, MA), and probed with the following primary antibodies (purchased from Santa Cruz Inc., Santa Cruz, CA): cyclin D1 (R-124, sc-6281), cyclin E (M-20, sc-481), p27 (C-19, sc-528), cyclin A (C-19, sc-596) and cyclin B1 (GNS1, sc-245). Blocking, antibody incubation and detection were performed using Western-StarTM Chemiluminescent Detection System (Tropix, Bedford, MA) according to the manufacturer's instructions.

Thymidine incorporation

The cells (1×10^5) were maintained in suspension over agarose as described above except for the use of 5 ml each of upper liquid and lower agarose medium in a 60-mm dish. Thirty min prior to harvesting the cells at each time point, the medium was supplemented with 5 μ Ci [methyl-³H]thymidine (specific activity 85 Ci/mmol). After the labelling period, the cells were harvested according to the procedure described above for isolation of total protein. This procedure was found to be suitable for precipitation and collection of DNA as well. The final pellet was neutralized with 25 μ l of 3 M Tris base, diluted with 25 μ l of water and the radioactivity measured.

RESULTS

Efficiency of formation of JB6 mouse epidermal cell colonies in soft agar

In promotion-sensitive P+ JB6 cells (clone 41.5a), TPA (10 ng/ml with 0.001% (vol/vol) DMSO as vehicle) induced the formation of large, anchorage-independent colonies in soft agar at a relatively high frequency (Figure 1). Vehicle alone did not induce a response that is more than 1% that with TPA. In contrast, promotion-resistant P-JB6 cells (clone 30.7a) exhibited a response in the presence of TPA that is only 4% that of P+ cells and the colonies were considerably smaller. In transformed Tx cells (clone RT101), a large number of colonies were formed in soft agar with vehicle alone.

It was previously reported that a noteworthy difference between P+ and P- cells involves activator protein-1 (AP-1) (Bernstein & Colburn 1989, Bernstein *et al.* 1991). AP-1 induces transcription of various genes in response to TPA in P+ but not in P- cells. In addition, Tx cells exhibit an elevated basal AP-1 activity in the absence of TPA (Dong, Lavrovsky & Colburn 1995). Analysis of the components of AP-1 complex has indicated that c-Jun, but not JunB, JunD, or c-Fos may be a limiting constituent in the P- cells (Bernstein, Bravo & Colburn 1992, Ben-Ari, Bernstein & Colburn 1992). Based on these and other observations, c-Jun/AP-1 is now considered to be required for promotion of neoplastic transformation (Dong *et al.* 1994b, Watts *et al.* 1995, Strickland *et al.* 1997).

Anchorage-independent growth of JB6 cells maintained in suspension over agarose

To initiate biochemical studies of the JB6 cells undergoing anchorage-independent growth, the cells were maintained in suspension in liquid medium over 0.7% agarose for 0–6 days. This period includes the previously reported 4-day TPA-commitment period—a reversible, TPA-dependent phase of transformation—during which JB6 cells must be continuously exposed to TPA to elicit formation of colonies in soft agar (Dion, Ginhart & Colburn 1988). Beyond this period TPA has little effect on colony formation—an irreversible, TPA-independent phase of transformation.

As shown in Figure 2a, TPA induced a significant increase in the amount of whole cell proteins in P+ (but not in P-) cells at 24 and 48 h after introduction of TPA. In contrast, in the absence of TPA (i.e. with vehicle alone), the levels of protein simply decreased during the entire 6-day period. In P- cells (with TPA), the protein levels remained approximately the same for the first 48 h and then declined. In Tx cells (with vehicle alone), the protein levels neither increased nor decreased. The TPA-induced increase in the amount of whole cell protein in P+ cells is similar to the previously reported stimulation of the incorporation of tritiated leucine into the protein of *in vivo* mouse epidermis following a single application of TPA (Baird, Sedgwick & Boutwell 1971). In the latter, the stimulation was induced promptly (within 6 h) and lasted for 2 to 4 days.

In the *in vivo* mouse epidermis TPA also induces an equally prompt and sustained stimulation of incorporation of tritiated cytidine into total RNA (Baird *et al.* 1971). Therefore, the amount of total RNA in JB6 cells was determined next (Figure 2b). We found that it closely paralleled that of total protein, suggesting that there may be a causal relationship between the two (De Young, Argyris & Gordon 1977, Argyris 1981, 1983, Hannan & Rothblum 1995)

Figure 1. Efficiency of formation of JB6 mouse epidermal cell colonies in soft agar. The promotion-sensitive P+ JB6 cells (clone 41.5a) (1×10^4) were exposed to DMSO (0.001% (vol/vol)) alone or TPA (10 ng/ml with 0.001% DMSO as vehicle); promotion-resistant P- cells (clone 30.7a) to TPA (10 ng/ml with 0.001% DMSO); and transformed Tx cells (clone RT101) to DMSO (0.001%) alone in soft agar (60-mm dish). Colony formation was scored at 14 days. The size of colonies counted was >8 cells. All assays were performed in triplicate.

and that c-Jun/AP-1 function may be essential for TPA-induced stimulation of both total RNA and protein syntheses.

Furthermore, we also found that TPA induced stimulation of the incorporation of tritiated thymidine into DNA in P+ (but not in P-) cells (Figure 2c). The stimulation started on or shortly before 24 h after introduction of TPA, peaked at about 48 h, and then declined to the control levels over the next several days. In the absence of TPA (P+ cells with vehicle alone) or in P- cells with TPA, incorporation of tritiated thymidine into DNA simply decreased with no stimulation during the 6-day period. In Tx cells (with vehicle alone), tritiated thymidine was incorporated into DNA at high levels during the entire 6-day period with maximum incorporation occurring at about 24 h. The TPA-induced stimulation of the incorporation of tritiated thymidine into DNA in P+ cells is strikingly similar to that in *in vivo* mouse epidermis following single application of TPA (Baird *et al.* 1971). In the latter, the stimulation started after a 12-h lag period, decreased transiently at about 24 h followed by a second increase between 24–48 h. The incorporation was the highest at 48 h and then declined to the control levels over the next 2–3 days. These results suggest that c-Jun/AP-1

Figure 2. Whole cell protein, total RNA, and incorporation of tritiated thymidine in JB6 cells maintained in suspension over agarose. The concentration of TPA and DMSO was the same as in Figure 1. They were applied in both upper liquid and lower agarose layers. The dishes were incubated at 37°C for 0–6 days in a humidified atmosphere of 5% CO₂ in air. (a) Whole cell protein. Approximately $5\text{--}8 \times 10^5$ cells were maintained in suspension (15 ml) over 0.7% agarose (15 ml) in a 100-mm dish. (b) Total RNA. The culture conditions were the same as for whole cell protein. (c) Incorporation of tritiated thymidine. Culture conditions were the same as for whole cell protein except that approximately 1×10^5 cells were suspended in 5 ml of upper liquid layer over 5 ml of lower agarose layer. At each time point, the cells were labelled with tritiated thymidine for 30 min prior to harvesting. ●, P+ (TPA, 10 ng/ml; DMSO, 0.001%); ■, P+ (DMSO, 0.001%); ○, P- (TPA, 10 ng/ml; DMSO, 0.001%); and □, Tx (DMSO, 0.001%).

Figure 2. Continued

function may also be indispensable for TPA-induced stimulation of DNA synthesis under anchorage-independent conditions.

Cyclins (A, B1, D1 and E) and p27Kip1

Anchorage-independent cells are generally arrested at the G1-S interphase and, therefore, unable to enter S phase and replicate their DNA (Guadagno & Assoian 1991, Guadagno *et al.* 1993, Han *et al.* 1993). These cells fail to produce cyclin A, an S phase specific cell-cycle regulatory protein (Guadagno *et al.* 1993, Zhu *et al.* 1996, Schulze *et al.* 1996, Spitkovsky *et al.* 1997). Enforced expression of cyclin A rescues entry into S phase in these cells (Guadagno *et al.* 1993). Even in the cells attached to a substratum, inhibition of cyclin A function through microinjection of plasmids encoding antisense cyclin A cDNA or affinity-purified anti-cyclin A antibody during G1 phase inhibits S phase entry (Girard *et al.* 1991, Pagano *et al.* 1992, Zindy *et al.* 1992). These results suggest that expression of cyclin A is required for anchorage-independent cells to enter S phase and replicate DNA.

To determine whether TPA induces cyclin A expression in P+ cells, Western blot analysis of cyclin A was performed using the cells maintained in suspension over agarose. As Figure 3a shows, TPA (with 0.001% DMSO as vehicle) stimulated the expression of cyclin A at 48 h when the levels of TPA-induced synthesis of DNA were also the highest. When the amount of DMSO was increased to 0.1%, TPA still stimulated the expression of cyclin A at 48 h, but the levels of pre-existing cyclin A at 24 h were considerably lower than those with 0.001% DMSO, suggesting that DMSO rapidly reduced the levels of pre-existing cyclin A. In the absence of TPA (P+ cells with 0.001% DMSO alone), no stimulation of cyclin A expression was observed during the entire 6-day period. In P- cells, TPA (with 0.001% DMSO) slightly stimulated the cyclin A expression at 96 h (Figure 3b). When the amount of DMSO was increased to 0.1%, a slight increase in cyclin A expression was again detected at 96 and 144 h. The levels of pre-existing cyclin A were however rapidly reduced in these cells. The slight increase in cyclin A expression at 96-144 h was apparently not sufficient to induce DNA synthesis (Figure 2c). These results suggest that P- cell mutation is slightly leaky and not entirely resistant to the promotion action of TPA. In Tx cells (with vehicle alone), the levels of cyclin A expression paralleled those of DNA synthesis (Figure 3b).

Cyclin B1 is a mitotic cyclin, the expression of which peaks after S phase/DNA synthesis. Figure 3a shows that TPA also induced the expression of cyclin B1 at 48 h in P+ (but not in P-) cells.

Cyclin D1 is a G1-phase specific cell-cycle regulatory protein which acts as a growth factor sensor to integrate extracellular signals with the cell cycle machinery (for a review article, see Sherr 1996). Synthesized as a delayed early gene in response to treatment of quiescent cells with mitogens, its continued synthesis depends on growth-factor stimulation regardless of the cell's position in the cycle.

As Figure 3a shows, TPA strongly stimulated the expression of cyclin D1 in P+ cells during the first 48 h. This stimulation was not significantly affected by increasing the amount of DMSO to 0.1% (data not shown). In the absence of TPA (P+ cells with 0.001% DMSO alone), cyclin D1 expression increased only slightly during the same 48-h period. In P- cells, TPA slowly stimulated the expression of cyclin D1 during the first 48 to 96 h (2 to 4 days) (Figure 3b). This slow accumulation of cyclin D1 may eventually cause the slight stimulation of the expression of cyclin A at 96-144 h.

Since stimulation of cyclin D1 gene expression by mitogens and other compounds depends on c-Jun/AP-1 binding site in its promoter (Herber *et al.* 1994, Albanese *et al.* 1995, Watanabe *et al.* 1996), it appears reasonable to assume that TPA stimulated the cyclin D1

Figure 3. Levels of cyclins (A, B1, D1 and E) and p27Kip1 proteins. (a) P+ JB6 cells maintained in suspension over agarose. The P+ JB6 cells ($5-8 \times 10^5$) were cultured in the presence of TPA (10 ng/ml) plus vehicle (DMSO, 0.001%) or vehicle alone. For the second row of cyclin A levels, P+ JB6 cells were cultured in the presence of TPA (10 ng/ml) plus 0.1% (rather than 0.001%) of DMSO. Protein extracts were analysed by Western immunoblotting and visualized using chemiluminescent detection procedure. (b) P- and Tx JB6 cells maintained in suspension over agarose. The P- JB6 cells ($5-8 \times 10^5$) were cultured in the presence of TPA (10 ng/ml) plus vehicle (DMSO, 0.001%), and the Tx JB6 cells ($5-8 \times 10^5$) in vehicle alone. For the second row of cyclin A levels, P- JB6 cells were cultured in the presence of TPA (10 ng/ml) plus 0.1% (rather than 0.001%) of DMSO.

expression through this site in P+ cells. In contrast, in P- cells which have reduced c-Jun/AP-1 function, TPA may have failed to stimulate the cyclin D1 expression although the P- mutation appeared to be a little leaky.

Cyclin E is another G1-phase specific cyclin which generally peaks at late G1 phase or G1-S interphase. As Figures 3a and 3b show, the levels of the expression of cyclin E did not correlate with those of either DNA synthesis or cyclin A expression.

p27Kip1 is a cyclin-dependent kinase inhibitor, the reduction of which is generally considered to be responsible for the activation of cyclin E-cyclin dependent kinase 2 (cdk2) complex and subsequent expression of cyclin A and S phase entry. Our results show that, like cyclin E, the levels of p27Kip1 did not correlate with those of either DNA synthesis or cyclin A expression.

Anchorage-independent growth of NMU38 rat mammary epithelial cells: effect of TPA and 17 β -oestradiol

NMU38 cells were originally established from well-differentiated primary mammary adenocarcinoma arising in a female Sprague-Dawley rat receiving single injection of MNU (Sukumar, Carney & Barbacid 1988, McKenzie *et al.* 1997). The tumour was dependent on ovarian hormones *in vivo*. NMU38 cells carry the activating G→A mutation in codon 12 of the Ha-ras-1 gene.

These cells, when maintained in suspension over agarose, required a combination of TPA and 17 β -oestradiol (5 ng/ml each) for maximum anchorage-independent growth (Figure 4a). The levels of DNA synthesis were more than additive with either TPA (10 ng/ml) or 17 β -oestradiol (10 ng/ml) alone. In the absence of either one of them, NMU38 cells did not grow to any significant extent. This latter observation was somewhat unexpected because these cells carry activated Ha-ras-1 mutation and also the fetal bovine serum (10% vol/vol) in the cell culture medium was not stripped of any steroid hormones. This phenotype was, however, maintained only during the first several passages when subcultured in the absence of 17 β -oestradiol. During the next 15 passages, the phenotype gradually shifted to that of transformed Tx JB6 cells, suggesting that, in the absence of 17 β -oestradiol, NMU38 cells underwent spontaneous transformation or preferential selection for this phenotype during this period, although they still retained the flat morphology.

Stimulation of the total RNA synthesis did not show any such synergistic effect with a combination of TPA and 17 β -oestradiol (Figure 4b).

17 β -Oestradiol was previously shown to activate cyclin D1 promoter through c-Jun/AP-1 site in oestrogen receptor (ER)-positive mammary epithelial cells (Altucci *et al.* 1996, Planas-Silva & Weinberg 1997, Prall *et al.* 1997). The 17 β -oestradiol-bound oestrogen receptor (ER) probably interacts directly with c-Jun/c-Fos heterodimer, thereby synergistically stimulating the transcription of c-Jun/AP-1-responsive genes including cyclin D1 (Gaub *et al.* 1990, Weisz & Rosales 1990, Philips, Chalbos & Rochefort 1993, Umayahara *et al.* 1994, Savouret *et al.* 1994, Astruc *et al.* 1995, Webb *et al.* 1995, Dumont *et al.* 1996, Kim, Brown & Birrer 1996, Uht *et al.* 1997, Paech *et al.* 1997).

Effects of calcitriol on TPA-induced anchorage-independent growth of P+ JB6 and NMU38 cells

Calcitriol (1 α ,25-dihydroxyvitamin D3) by itself was previously shown to neither induce nor suppress the colony formation of P+ JB6 cells (clone 41.5a) in soft agar, but when added together with TPA it enhanced the TPA-induced anchorage-independent growth of these cells in soft agar (Chang & Prince 1993).

Figure 4. Effect of 17β-oestradiol on TPA-induced stimulation of (a) the incorporation of triated thymidine and (b) total RNA in NMU38 cells maintained in suspension over agarose. Culture conditions were the same as described in the legend of Figure 2. ▼, TPA (5 ng/ml; DMSO, 0.0005%) plus 17β-oestradiol (5 ng/ml; DMSO, 0.0005%); ▲, 17β-oestradiol (10 ng/ml; DMSO, 0.001%); ■, TPA (10 ng/ml; DMSO, 0.001%) and ●, DMSO (0.001%).

Similar synergistic effect of calcitriol and TPA was observed with the incorporation of tritiated thymidine in P+ JB6 cells maintained in suspension over agarose (Figure 5a). Such effect, however, was not observed with total RNA (Figure 5b). Since calcitriol's cognate nuclear receptor (vitamin D3 receptor, VDR) and oestrogen receptor (ER) are part of the same steroid-thyroid hormone receptor superfamily, it is possible that calcitriol synergistically activated cyclin D1 promoter through c-Jun/AP-1 site in the same way as did 17 β -oestradiol.

In NMU38 cells, a combination of TPA and calcitriol induced only additive effect on DNA synthesis and calcitriol by itself was able to induce DNA synthesis to a significant extent in these cells (Figure 5c). Such effect was again not observed with total RNA (data not shown). It was previously reported that calcitriol is able to induce transcription of p21Cip1 gene in a VDR-dependent manner through a functional vitamin D response element (VDRE) in the p21Cip1 promoter (Liu *et al.* 1996). It was also reported that calcitriol is able to activate Raf-mitogen-activated protein kinase signalling pathway (Gniadecki 1996). Therefore, in NMU38 (but not in P+ JB6) cells which carry activated Ha-ras-1 mutation, calcitriol by itself may have increased the levels of c-Jun/AP-1 thereby inducing DNA synthesis.

Effects of all *trans*-retinoic acid, dexamethasone, ethanol and dimethyl sulfoxide (DMSO) on TPA-induced anchorage-independent growth of P+ JB6 cells

All *trans*-retinoic acid and glucocorticoids (e.g. fluocinolone acetonide, dexamethasone) have been known to prevent TPA-induced colony formation of P+ JB6 cells in soft agar (Colburn

Figure 5. Effect of calcitriol on TPA-induced stimulation of (a) the incorporation of tritiated thymidine and (b) total RNA in P+ JB6 cells, and (c) the incorporation of tritiated thymidine in NMU38 cells maintained in suspension over agarose. ▼, TPA (5 ng/ml; DMSO, 0.0005%) plus calcitriol (5 ng/ml; DMSO, 0.0005%); ▲, calcitriol (10 ng/ml; DMSO, 0.001%); ■, TPA (10 ng/ml; DMSO, 0.001%); and ●, DMSO (0.001%).

Figure 5. Continued

et al. 1981, Dong *et al.* 1994b, Lavrovsky *et al.* 1996). c-Jun/AP-1 transactivation, rather than the classical retinoic acid or glucocorticoid response element (RARE or GRE) transactivation, appears to be involved in this inhibition (Dong *et al.* 1995, Lavrovsky *et al.* 1996, Li *et al.* 1996, 1997). Ligand-bound glucocorticoid receptor (GR) is postulated to interact directly with c-Jun (Jonat *et al.* 1990, Yang-Yen *et al.* 1990). Ligand-bound retinoic acid receptor (RAR) may also interact with c-Jun either directly or indirectly (Yang-Yen *et al.* 1991, Soprano *et al.* 1996).

As Figure 6a shows, all *trans*-retinoic acid suppressed, in a dose-dependent manner, the TPA-induced anchorage-independent growth of P+ JB6 cells maintained in suspension over agarose. Vehicle alone (ethanol plus DMSO at final concentrations of 0.1% and 0.002%, respectively) did not significantly inhibit the TPA-induced stimulation of the incorporation of tritiated thymidine into DNA, but it rapidly suppressed the pre-existing synthesis of DNA. In contrast, all *trans*-retinoic acid inhibited TPA-induced stimulation of total RNA synthesis in a strange way; during the first 48 h TPA stimulated total RNA synthesis as if there was no all *trans*-retinoic acid added, but within next 24 h total RNA levels dropped rapidly to those typical of either P+ JB6 cells without TPA or P- JB6 cells with TPA (data not shown). Vehicle alone (0.1% ethanol plus 0.002% DMSO) did not inhibit TPA-induced stimulation of total RNA synthesis (data not shown).

A higher concentration of DMSO (0.1%) was found to inhibit TPA-induced colony formation of P+ JB6 cells in soft agar by approximately 25%. When these cells were maintained in suspension over agarose, 0.1% DMSO primarily suppressed the pre-existing synthesis of DNA in the same way as did 0.1% ethanol (data not shown). DMSO (0.1%) also rapidly suppressed the pre-existing cyclin A in these cells without significantly affecting the TPA-induced stimulation of cyclin A expression (Figures 3a and 3b). Like 0.1% ethanol, 0.1% DMSO did not inhibit TPA-induced stimulation of total RNA synthesis (data not shown).

Dexamethasone (0.1 μM) completely suppressed TPA-induced stimulation of the incorporation of tritiated thymidine in P+ JB6 cells (Figure 6b). The TPA-induced stimulation of total RNA synthesis, however, was only partially (by 50 to 60%) suppressed by 0.1 μM dexamethasone (data not shown).

DISCUSSION

Similarities between over-agarose suspension culture and traditional in-agar (soft agar) culture

It appears that the over-agarose suspension culture described here is operationally equivalent to the more traditional in-agar (soft agar) culture in terms of determining the TPA-induced anchorage-independent growth of JB6 cells.

First, in the promotion-sensitive P+ JB6 cells, TPA stimulated incorporation of tritiated thymidine (which peaks at 48 h) in over-agarose suspension culture and induced formation of colonies (after 14 days) in soft agar. In the absence of TPA, both of these events did not occur. Furthermore, in the promotion-resistant P- JB6 cells, both of these events again did not occur. In contrast, in the transformed Tx JB6 cells, both were highly elevated even though the cells were not exposed to TPA. Therefore, the TPA-induced stimulation of incorporation of tritiated thymidine in over-agarose suspension culture positively correlated with the TPA-induced formation of colonies in soft agar.

Further support comes from the studies of the effects of all *trans*-retinoic acid, dexamethasone, and calcitriol (1 α ,25-dihydroxyvitamin D3) on P+ JB6 cells. All *trans*-retinoic acid and

Figure 6. Effect of all *trans*-retinoic acid and dexamethasone on TPA-induced stimulation of the incorporation of tritiated thymidine in P+ JB6 cells maintained in suspension over agarose. (a) All *trans*-retinoic acid. ●, TPA (10 ng/ml; DMSO, 0.002%; ethanol, 0.1%); ■, TPA (10 ng/ml; DMSO, 0.001%) plus all *trans*-retinoic acid (0.05 μM; DMSO, 0.001%; ethanol, 0.1%); ▲, TPA (10 ng/ml; DMSO, 0.001%) plus all *trans*-retinoic acid (5 μM; DMSO, 0.001%; ethanol, 0.1%); and ▼, vehicle alone (DMSO, 0.002%; ethanol, 0.1%). (b) Dexamethasone. ●, TPA (10 ng/ml; DMSO, 0.002%); ■, TPA (10 ng/ml; DMSO, 0.001%) plus dexamethasone (0.1 μM; DMSO, 0.001%); and ▲, dexamethasone (0.1 μM; DMSO, 0.002%).

dexamethasone, both of which were previously reported to suppress TPA-induced formation of colonies in soft agar (Colburn *et al.* 1981, Dong *et al.* 1994b, Lavrovsky *et al.* 1996), also suppressed TPA-induced stimulation of incorporation of tritiated thymidine in over-agarose suspension culture, whereas calcitriol, which was previously reported to synergistically stimulate TPA-induced formation of colonies in soft agar (Chang & Prince 1993), also synergistically stimulated TPA-induced stimulation of incorporation of tritiated thymidine in over-agarose suspension culture.

Advantages and disadvantages of over-agarose suspension culture and traditional in-agar (soft agar) culture

One of the advantages of over-agarose suspension culture is that with this method it takes only several days to determine the effect of promoting agents and their inhibitors/stimulators, whereas with soft agar assay it takes 10 to 14 days. One point, however, bears noting. In the former, the TPA-induced stimulation of incorporation of tritiated thymidine peaks at 48 h, but it declines steadily over the next few days and, by the 6th day, there is almost no significant stimulation above control. It is unclear why incorporation of tritiated thymidine does not continue beyond 6 days. (In some cases, however, the 48-h peak is so strong that stimulation continues beyond 6 days.) In contrast, in the soft agar assay, each colony continues to proliferate for 14 days or longer.

Another advantage of over-agarose suspension culture is that, unlike soft agar culture, a large number of cells may be recovered almost quantitatively from the over-agarose suspension culture and subjected to various biochemical and molecular analyses.

One disadvantage of over-agarose suspension culture is that this method yields only semi-quantitative results. A case in point: 0.1% DMSO suppressed TPA-induced formation of P + JB6 cell colonies in soft agar by about 25%, but this extent of suppression was not detected using over-agarose suspension culture.

DNA synthesis, cyclins (A, B1, D1 and E) and p27Kip1

In P + JB6 cells, TPA-induced synthesis of DNA seems to begin at or shortly before 24 h, peaks at about 48 h, and then declines during the next several days until it falls to the control levels. This time interval coincides with the previously reported 4-day TPA-commitment period—a reversible, TPA-dependent phase of transformation—during which JB6 cells must be continuously exposed to TPA to elicit colony formation in soft agar (Dion *et al.* 1988). Beyond this period TPA has little effect on colony formation—an irreversible, TPA-independent phase of transformation. Since DNA synthesis is generally considered to be an irreversible event, TPA must first induce a chain of reversible molecular events which eventually lead to the irreversible event of DNA synthesis during this period.

Based on the results presented herein, we tentatively suggest the following sequence of events (Figure 7a). A key event in this sequence is the stimulation of c-Jun/AP-1 binding site in the cyclin D1 promoter (Altucci *et al.* 1996, Planas-Silva & Weinberg 1997, Prall *et al.* 1997). According to this suggestion, TPA first induces c-Jun/AP-1 (Bernstein & Colburn 1989, Bernstein *et al.* 1991), which in turn stimulates the transcription of cyclin D1 gene through this site. In the promotion-resistant P – JB6 cells, c-Jun is the limiting constituent of AP-1 (Bernstein & Colburn 1989, Bernstein *et al.* 1991). Therefore, these cells are unable to stimulate cyclin D1 expression. (In our hands, however, P – mutation turned out to be a little leaky and, as a result, a small stimulation of cyclin D1 expression was observed by Western blot analysis.) In transformed Tx JB6 cells, basal AP-1 activity is constitutively elevated

Figure 7. A proposed mechanism underlying the (a) TPA-induced growth of promotion-sensitive cells under anchorage-independent conditions and the (b) upstream cyclin D1 regulations.

(Dong *et al.* 1995) and, therefore, cyclin D1 expression may also be constitutively elevated in these cells.

Cyclin D1 is generally considered to form active complex with cyclin-dependent kinase 4 (cdk4) which phosphorylates retinoblastoma protein (Rb) (e.g. p107) (Zerfass-Thome *et al.* 1997, for a review article see Sherr 1996). Phosphorylation of Rb by cyclin D1-cdk4 complex leads to a dissociation of Rb from E2F/DP complexes and subsequent release of active E2F that controls cyclin A and other S-phase genes (Schulze *et al.* 1995, 1996). This pathway of cyclin D1-to-cyclin A transduction appears to be rather inefficient requiring a stronger stimulation of cyclin D1-cdk4 complex (such as ectopic overexpression of cyclin D1 (Schulze *et al.* 1996, Zhu *et al.* 1996)).

A more efficient way of stimulating the cyclin D1–cyclin A pathway appears to be through the activation of cyclin E-cdk2 complex which increases the levels of active E2F by positive-feedback loop (Botz *et al.* 1996, Geng *et al.* 1996). We found, however, that TPA does not induce stimulation of cyclin E expression nor inactivate p27Kip1, a cyclin-dependent kinase inhibitor. Similar observations were previously made in other cells (under adherent culture conditions) using other agents. For example, addition of 17 β -oestradiol to human mammary carcinoma cells relieved the cell cycle block created by tamoxifen or oestrogen antagonist treatment and increased the cyclin D1 levels significantly while the levels of cyclin E, cdk2, p27Kip1 and p21Cip1 were relatively constant (Planas-Silva and Weinberg 1997, Prall *et al.* 1997). In another example, ectopic expression of three D-type cyclins (D1, D2 and D3), but not cyclin E, rescued the defective mitogenic signalling in the mouse fibroblasts engineered to express a mutant CSF-1 receptor (Roussel *et al.* 1995). Paradoxically, cyclin E-cdk2 kinase complex was activated in many of these cases (Fang *et al.* 1996, Planas-Silva & Weinberg 1997). To reconcile this paradox it was proposed that the activation of cyclin E-cdk2 complex, without altering the levels of cyclin E or p27Kip1, may be achieved through redistribution of p21Cip1 from cyclin E-cdk2 complex to cyclin D1-cdk4 complex (Planas-Silva & Weinberg 1997). The redistributed p21Cip1 may increase the cdk4 kinase activity by promoting the assembly of the cyclin D1-cdk4 complex (LaBaer *et al.* 1997).

Finally, the activation of cyclin B1 has been reported to be dependent on cdk2 kinase activity associated with cyclin A (Moro *et al.* 1997).

Upstream cyclin D1 regulators

17 β -Oestradiol, calcitriol, all *trans*-retinoic acid and glucocorticoids appear to control TPA-induced anchorage-independent growth of promotion-sensitive cells at the level of c-Jun/AP-1 binding site in the cyclin D1 promoter (Figure 7b).

First, there is now increasing evidence that the 17 β -oestradiol-bound oestrogen receptor (ER) interacts directly with c-Jun/c-Fos heterodimer through this site (also called half-palindromic oestrogen response element (ERE)) in the cyclin D1 promoter, thereby synergistically stimulating the transcription of cyclin D1 gene (Gaub *et al.* 1990, Weisz & Rosales 1990, Philips *et al.* 1993, Umayahara *et al.* 1994, Savouret *et al.* 1994, Astruc *et al.* 1995, Webb *et al.* 1995, Dumont *et al.* 1996, Kim *et al.* 1996, Uht *et al.* 1997, Paech *et al.* 1997). This site is apparently different from the classical palindromic ERE where ligand-bound ER directly binds as a homodimer.

Second, although there is no report in the literature supporting the contention that calcitriol-bound vitamin D3 receptor (VDR) (for recent review articles, see Strugnelli & Deluca 1997, Christakos *et al.* 1996) interacts with c-Jun/c-Fos heterodimer through this site, since VDR and ER are part of the same steroid-thyroid hormone receptor superfamily, it is possible that calcitriol synergistically activates cyclin D1 promoter through c-Jun/AP-1 site in

the same way as 17β -oestradiol. The retinoid X receptor (RXR) may participate only in the classical vitamin D response element (VDRE) and not in this interaction.

Lastly, it is generally assumed that the ligand-bound retinoic acid receptor (RAR) or glucocorticoid receptor (GR) inhibits the transcription of cyclin D1 gene through this site by directly or indirectly interacting with c-Jun/c-Fos heterodimer, thereby suppressing TPA-induced anchorage-independent growth of the promotion-sensitive cells (Dong *et al.* 1995, Lavrovsky *et al.* 1996, Li *et al.* 1996, 1997). Again, the retinoid X receptor (RXR) may participate only in the classical retinoic acid response element (RARE) and not in this interaction (Li *et al.* 1996, 1997). Also, this site is different from the classical glucocorticoid response element (GRE) where ligand-bound GR directly binds as a homodimer (Dong *et al.* 1995).

We found that DMSO (0.1%) or ethanol (0.1%) did not so much inhibit the TPA-induced stimulation of cyclin D1, cyclin A and DNA synthesis as the pre-existing cyclin A and DNA synthesis. The latter two declined rapidly in the presence of either of these agents, whereas the pre-existing cyclin D1 was not significantly affected by these agents. Therefore, it appears as if DMSO or ethanol actively and specifically destroyed pre-existing cyclin A. We also found that all *trans*-retinoic acid ($0.05\ \mu\text{M}$) has dual activities; it not only inhibited the TPA-induced stimulation of DNA synthesis but also suppressed the pre-existing synthesis of DNA.

TPA-induced stimulation of total RNA synthesis

The synthesis of ribosomal RNA (rRNA) constitutes approximately 40% to 60% (up to 80% in rapidly growing cells) of the total cellular RNA synthesis and at least 90% of the existing ribosomes are already engaged in synthesizing protein (for a review article, see Hannan & Rothblum 1995). Therefore, even a small increase in rRNA synthesis can result in a significant increase in the total cellular protein synthetic capacity (Hannan & Rothblum 1995).

Based on these considerations, it is reasonable to assume that TPA induced rRNA synthesis (Chao & Pellegrini 1993, Hanan & Rothblum 1995) in P+ JB6 and NMU38 cells under anchorage-independent culture conditions. Since TPA failed to induce total RNA synthesis in P- JB6 cells, it is likely that this induction is dependent on c-Jun/AP-1 function. What is not clear in this case is the identity of the dimeric partner of c-Jun because neither 17β -oestradiol nor calcitriol exhibited any synergistic effect on TPA-induced total RNA synthesis and the inhibition of total RNA synthesis by all *trans*-retinoic acid and dexamethasone was considerably smaller than that of DNA synthesis. Ligand-bound nuclear receptors may not be able—partially or entirely—to interact with c-Jun if its heterodimeric partner is something other than c-Fos (Gaub *et al.* 1990, Paech *et al.* 1997).

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