[CANCER RESEARCH 53, 2217–2220, May 15, 1993]

Advances in Brief

1α,25-Dihydroxyvitamin D_3 Enhances 12-*O*-Tetradecanoylphorbol-13-acetate-induced Tumorigenic Transformation and Osteopontin Expression in Mouse JB6 Epidermal Cells[1]

Pi-Ling Chang and Charles W. Prince[2]

Department of Nutrition Sciences [P-L. C., C. W. P.] and Comprehensive Cancer Center [C. W. P.], University of Alabama at Birmingham, Birmingham, Alabama 35294

Abstract

To study the role of 1α,25-dihydroxyvitamin D_3 (calcitriol) in tumor promotion, we used JB6 C141.5a cells, a mouse epidermal cell model of tumor promotion. The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) irreversibly induces anchorage-independent growth and tumorigenicity in these cells. Since we previously showed that calcitriol does not transform these cells but inhibits their proliferation, we hypothesized that calcitriol would inhibit TPA-induced transformation. Concurrent treatment of JB6 C141.5a cells with TPA and calcitriol revealed that calcitriol enhanced (1.7- to 10-fold, depending on dose) TPA-induced anchorage-independent growth without enhancing cell proliferation. Furthermore, a more than additive effect on osteopontin mRNA and protein levels was observed with concurrent drug treatment, which yielded a more highly phosphorylated form of osteopontin. These studies suggest coordinate regulation between the signaling pathways for calcitriol and TPA in JB6 C141.5a cells and further implicate expression of phosphorylated osteopontin in tumorigenesis.

Introduction

Several studies suggest a role for the hormonal form of vitamin D, 1α,25-dihydroxyvitamin D_3 or calcitriol, in modulating cell differentiation, cell proliferation, and tumor promotion. Suda *et al.* (1) and others (2, 3) have shown that calcitriol induces mouse (M1) and human leukemia cells (HL-60) to differentiate and concurrently reduces cell proliferation. Calcitriol also induces primary mouse epidermal cells to differentiate into cornified cells (4). Furthermore, high concentrations of calcitriol decrease proliferation of some cancer cell lines such as breast cancer cells (5) and malignant melanomas (6). In addition to modulating cell proliferation and differentiation, calcitriol also has a role in tumor promotion. Like the tumor promoter TPA,[3] calcitriol has been shown to enhance tumorigenic transformation in carcinogen-treated cells *in vitro* (7–9). *In vivo*, calcitriol applied to CD-1 mouse skin 1 h before or a day after induction with DMBA also stimulated tumor formation by five-fold compared to animals treated with only DMBA (10). Besides enhancing tumor formation, calcitriol has also been shown to inhibit tumor formation. Treatment of DMBA-initiated CD-1 mouse skin with a topical application of calcitriol 1 h before the application of TPA resulted in 50% inhibition of tumor formation (11). Similar studies using DMBA-initiated Sencar mouse skin also showed that application of calcitriol prior to addition of TPA greatly inhibited tumor formation (12).

These contradictory studies on the role of calcitriol in modulating tumor promotion indicate the need for further investigation. We chose a mouse epidermal cell line, JB6 C141.5a, to investigate the role of calcitriol in tumor promotion. These cells were cloned from parental, nonclonal JB6 cells derived by Colburn *et al.* (13) from dimethylsulfoxide-treated epidermal cells of BALB/c mice. The parental JB6 and C141.5a cells respond to TPA with irreversible induction of anchorage-independent growth and tumorigenic transformation (14, 15). Since our previous observations indicated that calcitriol does not induce tumorigenic transformation of JB6 C141.5a cells but reduces their rate of proliferation (16), we hypothesized that calcitriol would inhibit TPA-induced tumorigenic transformation of these cells. In addition, we examined the production of OPN by JB6 C141.5a cells treated with both calcitriol and TPA. OPN, originally isolated as a bone matrix phosphoprotein, has since been found in other tissues. Several tumorigenic and/or metastatic cell lines have increased expression of phosphorylated OPN protein or mRNA compared to paired, nontumorigenic counterparts (17–21). Furthermore, *ras*-transformed NIH 3T3 cells not only synthesize and secrete elevated amounts of OPN compared to nontransformed cells, but they also exhibit enhanced adhesion to phosphorylated OPN (22).

We report here that calcitriol enhanced, rather than inhibited, TPA-induced tumorigenic transformation of JB6 C141.5a cells. Concurrent addition of calcitriol and TPA to these cells induced elevated secretion of phosphorylated OPN compared to TPA-treated cells. Immunoadsorption studies and Northern blot analyses also indicated that OPN protein and steady-state levels of OPN mRNA were significantly elevated in cells treated with TPA and calcitriol *versus* those treated with TPA alone.

Materials and Methods

Materials. TPA was from Calbiochem, San Diego, CA. 1α,25-dihydroxyvitamin D_3 was purchased from Biomol Research Laboratories, Plymouth Meeting, PA. MEM and antibiotics were from Mediatech, Inc., Herndon, VA. FBS was from HyClone Laboratories, Inc., Logan, UT. Carrier-free $^{32}PO_4$ (8 mCi/Ml), and [^{35}S]-methionine (1101 Ci/mmol) were from ICN Radiochemicals, Irvine, CA [α-^{32}P]dCTP and Gene Screen Plus were from NEN-DuPont, Wilmington, DE. All other reagents were of the highest grade available.

Cell Culture. Mouse epidermal JB6 C141.5a cells (a gift from Dr. N. H. Colburn, National Cancer Institute, Frederick, MD) were maintained in MEM containing 5% heat-inactivated FBS, 2% L-glutamine, and 0.5% antibiotics. Cells were subcultured at near confluence (about twice a week). Monthly examination of the cells for Mycoplasma by DNA fluorochrome staining showed no contamination.

Anchorage-independent Growth in Agar. The detailed procedure for this assay was described previously (23). Generally, a base layer of 0.5% agar (7 ml) containing dimethyl sulfoxide as control, calcitriol, TPA, or calcitriol and TPA was plated onto 60-mm Petri dishes. Then, 10,000 cells, suspended in 1.5 ml of 0.3% agar containing the appropriate treatment, were poured on top of the solidified base layer. Petri dishes were incubated at 37°C for 14 days in a

Received 3/1/93; accepted 4/2/93.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This investigation was supported by NIH Grant DE06739 and a Research Career Development Award (DE00247) to C.W.P. and a Clinical Nutrition Research Unit center grant from the National Cancer Institute to the Department of Nutrition Sciences (Dr. C. L. Krumdieck, P.I.).

[2] To whom requests for reprints should be addressed, at Department of Nutrition Sciences, Webb Nutrition Sciences Building, 1675 University Boulevard, University of Alabama at Birmingham, Birmingham, AL 35294.

[3] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SDS, sodium dodecyl sulfate; OPN, osteopontin; DMBA, dimethylbenz(*a*)anthracene; MEM, Eagle's minimum essential medium; cDNA, complementary DNA; SSC, standard saline-citrate; FBS, fetal bovine serum.

humidified atmosphere of 5% CO_2 in air. Clusters with at least 10 cells were counted as colonies. In every experiment, each treatment was tested in triplicate.

Metabolic Labeling of Cell Cultures. JB6 C141.5a cells seeded at 20,000 cells/cm^2 were grown in MEM with 5% heat-inactivated FBS and 0.5% antibiotics to a near confluent state prior to treatment with vehicle (dimethyl sulfoxide), calcitriol, TPA or both. At the beginning and end of treatment, cell density was determined. During the last 4 h of drug incubation, cell medium was changed to methionine- or phosphate-free medium, as appropriate, for 1 h. [^{35}S]methionine (100 μCi/ml) or $^{32}PO_4$ (100 μCi/ml) was then added to methionine- or phosphate-free medium, respectively, and cells were incubated for 3 h. Medium collected for determination of total protein by trichloroacetic acid precipitation was first diluted with an equal volume of phosphatase/protease inhibitors (10 mM levamisole, 200 mM 6-aminocaproic acid, and 2 mM phenylmethylsulfonyl fluoride). Medium collected for immunoadsorption assay was diluted with 50 mM Tris-HCl buffer, pH 8.0, containing 0.4 M NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.02% NaN_3, and 2 mM phenylmethylsulfonyl fluoride. The mixture was centrifuged to remove cell debris.

Immunoadsorption. The procedures were described previously (16). Briefly, radiolabeled medium was incubated for 2 h at 4°C with protein A-Sepharose 6MB bound to preimmune antibody. The supernatant, after centrifugation was incubated with anti-osteopontin antiserum [raised against purified osteopontin from rat bone (24)] for 4 h. Then 100 μl of 10% protein A-Sepharose was added to the supernatant and incubated overnight at 4°C. The pellet after centrifugation was washed 3 times with 0.1 M Tris-HCl, pH 7.4, containing 0.15 M NaCl, 0.25% deoxycholate, and 0.1% Nonidet P-40, twice with Dulbecco's phosphate-buffered saline, and once with glass-distilled deionized water. The sample was heated for 5 min at 95°C with 0.1 M Tris-HCl, pH 6.8, containing 5% mercaptoethanol and 2% SDS, centrifuged, and analyzed by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels. The gel was stained with Coomassie brilliant blue, destained, dried for autoradiography, or treated for fluorography.

Northern Blot Analysis. The procedure for extraction of RNA has been described (25). The integrity of total RNA was determined by electrophoresis on a 0.7% agarose gel containing ethidium bromide. Undegraded RNA samples were used for Northern blot analysis. Total RNA was fractionated on 1.1% agarose gel containing 2.2 M formaldehyde and transferred to Gene Screen Plus membrane using 10 × SSC (1.5 M NaCl and 0.15 M sodium citrate) as the transfer medium. The procedure for hybridization of RNA was as described in the manual from NEN-DuPont. Briefly, the membrane was prehybridized in 10 ml of 50% formamide, 1% SDS, 10% dextran sulfate, and 1 M sodium chloride for 15 min at 42°C. Osteopontin cDNA (a gift from D. Denhardt, Rutgers University, NJ) was radiolabeled using the Prime It random primer kit and [α-^{32}P]dCTP (3000 Ci/mmol). Radiolabeled cDNA was purified from the unincorporated [α-^{32}P]dCTP using NucTrap push column. Denatured ^{32}P-labeled osteopontin cDNA and 250 μl of denatured salmon testes DNA were added to the membrane with hybridization solution at 42°C for 16 h. After hybridization, the membrane was washed twice with 100 ml of 2 × SSC for 5 min, twice with 200 ml of 2 × SSC and 1% SDS at 60°C for 30 min, and twice with 100 ml of 0.1 × SSC for 30 min. Without drying, the membrane was exposed to Kodak X-OMAT film in a cassette with intensifying screens at −20°C.

Results and Discussion

Since calcitriol does not induce tumorigenic transformation but does inhibit the proliferation of JB6 C141.5a cells (16), we hypothesized that calcitriol would inhibit TPA-induced tumorigenic transformation of these cells. Our results, however, are contrary to our hypothesis. An anchorage-independent growth assay was used to assess tumorigenic transformation of JB6 C141.5a cells treated with TPA and calcitriol. Fig. 1*A* shows that the addition of calcitriol (5 ng/ml) concurrently with varying concentrations of TPA (0.05–500 ng/ml) enhanced colony formation 1.7- to 10-fold more than TPA (0.05–500 ng/ml) alone. Also, varying doses of calcitriol (0.05–500 ng/ml), which normally do not induce tumorigenic transformation of JB6 C141.5a cells, induced colony formation in the presence of TPA (5 ng/ml) to a greater extent than in cells treated with TPA only (Fig. 1, *A* and *B*). These results indicated that even though calcitriol by itself does not have the capacity to induce colony formation, it has the ability to enhance TPA-induced tumorigenic transformation of JB6 C141.5a cells. Besides an increase in colony formation, we found that colony size was also increased in calcitriol and TPA-treated cells compared to TPA-treated cells (data not shown). Hosoi *et al.* (7) have shown that calcitriol enhanced TPA-induced tumorigenic transformation of parental, nonclonal JB6 cells. However, in these cells calcitriol promoted anchorage-independent growth and cell proliferation, in contrast to our findings in the C141.5a cells (16).

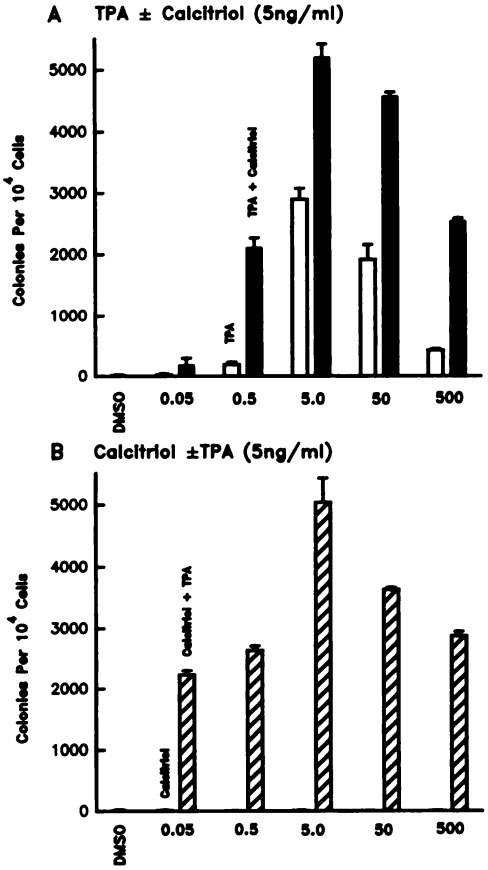

Fig. 1. Anchorage-independent growth of JB6 C141.5a cells treated with TPA, calcitriol, or TPA with calcitriol. Ten thousand cells were suspended in 0.3% agar and layered on a base layer of 0.5% agar with both layers containing vehicle (*A*; 0.001% dimethyl sulfoxide), TPA (0.05–500 ng/ml, open column), or TPA (0.05–500 ng/ml) with calcitriol (5 ng/ml; ■) or containing vehicle (*B*; 0.001% dimethyl sulfoxide), calcitriol (0.05–500 ng/ml) or calcitriol (0.05–500 ng/ml) with TPA (5 ng/ml; □). Cells were incubated for 14 days in 37°C incubator. Colonies containing clusters of at least 10 cells were counted. Each treatment was done in triplicate. Cells were from passage 34.

To determine if calcitriol-enhanced, TPA-induced colony formation could be due to effects on cell proliferation, a cell growth study was performed. Fig. 2 shows that after 3 days of treatment the rate of cell proliferation was slower in cells incubated with calcitriol (10 ng/ml) and TPA (10 ng/ml), concurrently, than in cells treated with TPA alone. Therefore, the enhancement of TPA-induced tumorigenic transformation by calcitriol is not due to stimulation of cell proliferation. Morphological alterations characteristic of TPA-treated JB6 C141.5a cells (16) were also observed in cells treated with both drugs (data not shown).

In addition to enhancement of anchorage-independent growth and morphological changes, we also observed an increase in expression of OPN mRNA and secretion of phosphorylated OPN by JB6 C141.5a cells treated concurrently with TPA and calcitriol. Autoradiography and densitometric analysis of TCA-precipitated $^{32}PO_4$-labeled OPN analyzed by 10% SDS-polyacrylamide gel electrophoresis indicated

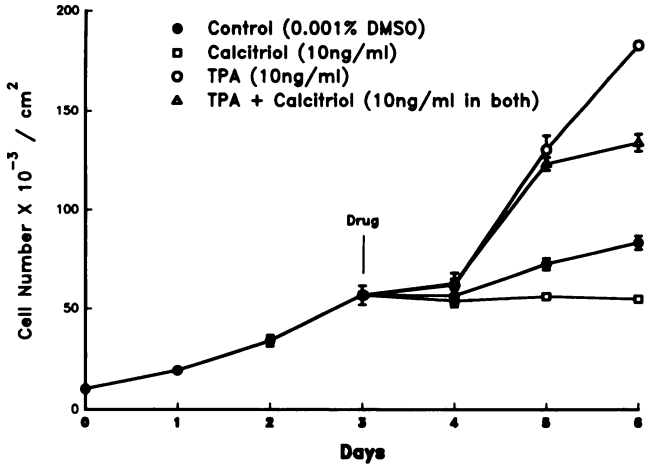

Fig. 2. Growth of JB6 C141.5a cells treated with TPA, calcitriol, or TPA with calcitriol. Cells were seeded at 10,000 cells/cm^2 in 12-well plates and grown for 3 days to near confluent state. Drugs were added to medium containing 5% FBS, 2% glutamine, and 0.5% antibiotics for 3 days: 0.001% dimethyl sulfoxide (●); 10 ng/ml calcitriol (□); 10 ng/ml TPA (○); calcitriol and TPA both at 10 ng/ml (△). Each day cells were trypsinized and counted using a Coulter counter. Triplicate samples were done for each treatment.

Table 1 *Stimulation of expression of OPN protein and mRNA by TPA, calcitriol, or both in JB6 Cl41.5a cells*

OPN	Time (h)	Control[a]	TPA[a,b]	Calcitriol[a,b]	TPA + Calcitriol[a,b]
^{35}S-protein	24	1	32	6	56
mRNA	24	1	38	10	70
^{35}S-protein	48	1	37	9	54

[a] Values obtained from densitometric analysis of fluorogram of immunoadsorbed OPN and of autoradiogram of Northern analyses of OPN mRNA (see legend to Fig. 3). Cells were treated with dimethyl sulfoxide 0.001% as control, TPA (10 ng/ml), calcitriol (10 ng/ml), or both at (10 ng/ml) for 24 or 48 h and then analyzed as described in "Materials and Methods."

[b] Ratio to control.

that cells treated with 10 ng/ml of TPA and calcitriol concurrently for 24 or 48 h (not shown) secreted 1.6- and 2.3-fold more phosphorylated OPN, respectively, than cells treated with only TPA (Fig. 3*A*). As in prior studies, treatment with calcitriol alone did not induce phosphorylated OPN (16). Use of a similar treatment protocol and [^{35}S]-methionine labeling and immunoadsorption analyses showed that 24 (Fig. 3*B*) or 48 h (not shown) treatment with TPA and calcitriol induced 1.7- and 1.5-fold increases, respectively, in secreted OPN protein compared to treatment with TPA alone (Table 1). Comparing the ratio of $^{32}PO_4$-labeled OPN to [^{35}S]methionine-labeled OPN after 24 h and 48 h of concurrent treatment with TPA and calcitriol (1.6/1.7 and 2.3/1.5, respectively), shows that after 48 h of treatment the JB6 cells were secreting OPN phosphorylated to a greater extent, on average, than cells treated only with TPA. Thus, calcitriol, while not able to induce synthesis of phosphorylated OPN, can enhance the synthesis and secretion of phosphorylated OPN induced by TPA. Examination of steady-state levels of OPN mRNA in JB6 C141.5a cells treated with calcitriol, TPA, or TPA and calcitriol concurrently showed that the combination produced much greater than an additive effect (Fig. 3*C*; Table 1).

Previously, we showed that calcitriol induced the synthesis and secretion of nonphosphorylated OPN by JB6 C141.5a cells but did not transform them. TPA, however, induced secretion of phosphorylated OPN and induced tumorigenic transformation (16). These results suggested that phosphorylation of OPN may play an important factor in determining tumorigenic transformation of these cells. Our current findings support this suggestion. Calcitriol not only enhanced TPA-induced tumorigenic transformation of JB6 C141.5a cells but also enhanced the secretion of phosphorylated OPN. Furthermore, Northern blot analysis and [^{35}S]methionine-labeling indicated that addition of both calcitriol and TPA to JB6 C141.5a cells enhanced the level of

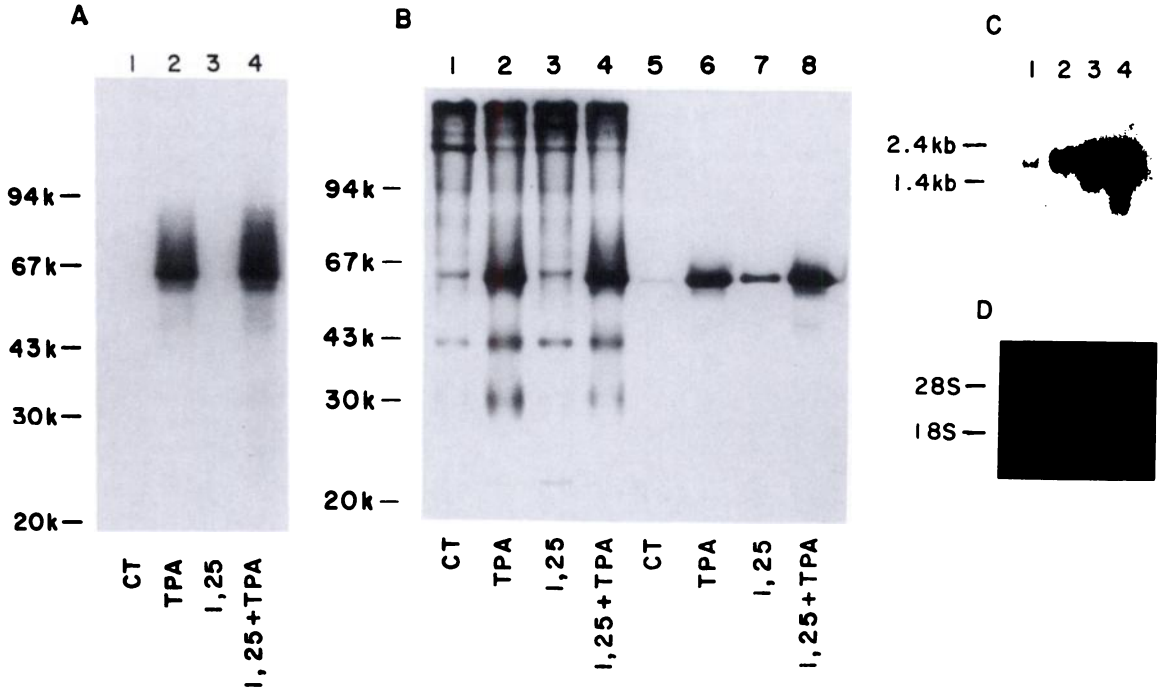

Fig. 3. Expression of OPN protein and mRNA. *A*, autoradiogram of 10% SDS-Polyacrylamide gel electrophoresis of $^{32}PO_4$-labeled OPN secreted by JB6 C141.5a treated with TPA, calcitriol, or TPA with calcitriol for 24 h. Labeled medium collected was TCA-precipitated and washed 3 times with 10% TCA and once with ethanol. Nonreducing sample buffer was added to the sample, heated for 10 min, and then run on 10% SDS-Polyacrylamide gel electrophoresis. *Lane 1*, 0.001% dimethyl sulfoxide; *Lane 2*, 10 ng/ml TPA; *Lane 3*, 10 ng/ml calcitriol; *Lane 4*, TPA and calcitriol both at 10 ng/ml. *B*, fluorogram of 10% SDS-Polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled proteins secreted by JB6 C141.5a treated with TPA, calcitriol, or both drugs for 24 h. *Lanes 1–4*, TCA precipitated proteins; *Lanes 5–8*, immunoadsorbed OPN. *Lanes 1* and *5*, 0.001% dimethyl sulfoxide; *Lanes 2* and *6*, 10 ng/ml TPA; *Lanes 3* and *7*, 10 ng/ml calcitriol; *Lanes 4* and *8*, TPA and calcitriol, both at 10 ng/ml. *C*, autoradiogram of Northern blot from JB6 C141.5a cells treated with TPA, calcitriol, or both drugs for 24 h. Cells were incubated with 0.001% dimethyl sulfoxide (*Lane 1*), 10 ng/ml calcitriol (*Lane 2*), 10 ng/ml TPA (*Lane 3*), or TPA and calcitriol, both at 10 ng/ml (*Lane 4*). Ten μg of total RNA from each sample were run on a denatured agarose gel transferred to Gene Screen Plus membrane, and probed with [α-^{32}P]dCTP-labeled OPN cDNA (5.2 × 10^8 cpm/μg) as described in "Materials and Methods." *D*, ethidium bromide staining of ribosomal RNA from the membrane described in *C*.

steady-state OPN mRNA and its secreted protein by about 2-fold when compared to that of TPA treated cells. These enhanced levels were not simply due to additive effects of calcitriol and TPA.

Our results also indicate that some form of cooperativity or overlap exists between the signaling pathways utilized by TPA and calcitriol. Since the mouse OPN promoter has both AP-1 sites (26) and a vitamin D response element (27), it may be that interacting transcription factors regulate OPN expression as well as that of other genes involved in the tumorigenic transformation of JB6 C141.5a cells.

Acknowledgments

We thank Dr. David Denhardt for the gift of the OPN cDNA insert. We also thank William H. Vaughn for the illustrations, Glenda Harris for typing the manuscript, and Diccie Dickie for Mycoplasma testing of the JB6 cells.

References

1. Suda, T., Miyaura, C., Abe, E., and Kuroki, T. Modulation of cell differentiation, immune responses and tumor promotion by vitamin D compounds. *In:* W. A. Peck (ed.), Bone and Mineral Research, pp. 1–45. New York: Elsevier Science Publishers, 1986.
2. Haskovec, C., Lemez, P., Neuwirtova, R., Wilhelm J., and Jarolim P. Differentiation of human myeloid leukemia cell line ML-1 induced by retinoic acid and 1,25-dihydroxyvitamin D_3 Neoplasma (Bratisl.), *37:* 565–572, 1990.
3. McCarthy, D. M., San Miguel, J. F., Freake, H. C., Green, P. M., Zola, H., Catovsky, D., and Goldman, J. M. 1,25-Dihydroxyvitamin D_3 inhibits proliferation of human promyelocytic leukemia (HL60) cells and induces monocyte-macrophage differentiation in HL60 and normal human bone marrow cells. Leukemia Res., *7:* 51–55, 1983.
4. Hosomi, J., Hosoi, J., Abe, E., Suda, T., and Kuroki, T. Regulation of terminal differentiation of cultured mouse epidermal cells by 1α,25-dihydroxyvitamin D_3. Endocrinology, *3:* 1950–1957, 1983.
5. Frampton, R. J., Omond, S. A., and Eisman, J. A. Inhibition of human cancer cell growth by 1,25-dihydroxyvitamin D_3 metabolites. Cancer Res., *43:* 4443–4447, 1983.
6. Colston, K., Colston, M. J., and Feldman, D. 1α,25-Dihydroxyvitamin D_3 and malignant melanoma: the presence of receptors and inhibition of cell growth in culture. Endocrinology, *108:* 1083–1086, 1981.
7. Hosoi, J., Abe, E., Suda, T., Colburn, N. H., and Kuroki, T. Induction of anchorage-independent growth of JB6 mouse epidermal cells by 1α,25-dihydroxyvitamin D_3. Cancer Res., *46:* 5582–5586, 1986.
8. Jones, C. A., Callaham, M. F., and Huberman, E. Enhancement of chemical carcinogen-induced cell transformation in hamster embryo cells by 1α,25-dihydroxycholecalciferol, the biologically active metabolite of vitamin D_3. Carcinogenesis (Lond.), *5:* 1155–1159, 1984.
9. Kuroki, T., Sasaki, K., Chida, K., Abe, E., and Suda, T. 1α,25-dihydroxyvitamin D_3 markedly enhances chemically induced transformation in BALB 3T3 cells. Gann, *74:* 611–614, 1983.
10. Wood, A. W., Chang, R. L., Huang, M-T., Baggiolini, E., Partridge, J. J., Uskokovic, M., and Conney, A. H. Stimulatory effect of 1α,25-dihydroxyvitamin D_3 on the formation of skin tumors in mice treated chronically with 7,12-dimethylbenz[*a*]-anthracene. Biochem. Biophys. Res. Commun., *130:* 924–931, 1985.
11. Wood, A. W., Chang, R. L., Uskokovic, M., and Conney, A. H. 1α,25-dihydroxyvitamin D_3 inhibits phorbol ester-dependent chemical carcinogenesis in mouse skin. Biochem. Biophys. Res. Commun., *116:* 605–611, 1983.
12. Chida, K., Hashiba, H., Fukushima, M., Suda, T., and Kuroki, T. Inhibition of tumor promotion in mouse skin by 1α,25-dihydroxyvitamin D_3. Cancer Res., *45:* 5426–5430, 1985.
13. Colburn, N. H., Bruegge, W. F. V., Bates, J. R., Gray, R. H., Rossen, J. D., Kelsey, W. H., and Shimada, T. Correlation of anchorage-independent growth with tumorigenicity of chemically transformed mouse epidermal cells. Cancer Res., *38:* 624–634, 1978.
14. Colburn, N. H., Former, B. F., Nelson, K. A., and Yuspa, S. H. Tumor promoter induces anchorage independence irreversibly. Nature (Lond.), *281:* 589–591, 1979.
15. Colburn, N. H., Koehler, B. A., and Nelson, K. J. A cell culture assay for tumor-promoter-dependent progression toward neoplastic phenotype: detection of tumor promoters and promotion inhibitors. Teratog. Carcinog. Mutagen., *1:* 87–96, 1980.
16. Chang, P. L., and Prince, C. W. 1α,25-Dihydroxyvitamin D_3 stimulates synthesis and secretion of nonphosphorylated osteopontin (secreted phosphoprotein 1) in mouse JB6 epidermal cells. Cancer Res., *51:* 2144–2150, 1991.
17. Craig, A. M., Bowden, G. T., Chambers, A. F., Spearman, M. A., Greenberg, A. H., Wright, J. A., McLeod, M., and Denhardt, D. T. Secreted phosphoprotein mRNA is induced during multi-stage carcinogenesis in mouse skin and correlates with the metastatic potential of murine fibroblasts. Int. J. Cancer, *46:* 133–137, 1990.
18. Craig, A. M., Nemir, M., Mukherjee, B. B., Chambers, A. F., and Denhardt, D. T. Identification of the major phosphoprotein secreted by many rodent cell lines as 2ar/osteopontin: enhanced expression in H-*ras*-transformed 3T3 cells. Biochem. Biophys. Res. Commun., *157:* 166–173, 1988.
19. Craig, A. M., Smith, J. H., and Denhardt, D. T. Osteopontin, a transformation-associated cell adhesion phosphoprotein, in induced by 12-*O*-tetradecanoylphorbol 13-acetate in mouse epidermis. J. Biol. Chem., *264:* 9682–9689, 1989.
20. Senger, D. R., Perruzzi, C. A., and Papadopoulos, A. Elevated expression of secreted phosphoprotein 1 (osteopontin, 2ar) as a consequence of neoplastic transformation. Anticancer Res., *9:* 1291–1300, 1989.
21. Smith, J. H., and Denhardt, D. T. Molecular cloning of tumor promoter-inducible mRNA found in JB6 mouse epidermal cells: induction is stable at high, but not at low, densities. J. Cell. Biochem., *34:* 13–22, 1987.
22. Chambers, A. F., Hota, C., and Prince, C. W. Adhesion of metastatic, *ras*-transformed NIH 3T3 cells to osteopontin, fibronectin, and laminin. Cancer Res., *53:* 701–706, 1993.
23. MacPherson, I. Agar suspension culture for quantitation of transformed cells. *In:* K. Habel and N. P. Salzman (eds.), Fundamental Techniques in Virology, pp. 214–219. New York: Academic Press, 1969.
24. Prince, C. W., Oosawa, T., Butler, W. T., Tomana, M., Bhown, A. S., Bhown, M., and Schrohenloher, R. E. Isolation, characterization, and biosynthesis of a phosphorylated glycoprotein from rat bone. J. Biol. Chem., *262:* 2900–2907, 1987.
25. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162:* 156–159, 1987.
26. Craig, A. M., and Denhardt, D. T. The murine gene encoding secreted phosphoprotein 1 (osteopontin): promoter structure, activity, and induction *in vivo* by estrogen and progesterone. Gene (Amst.), *100:* 163–171, 1991.
27. Noda, M., Vogel, R. L., Craig, A. M., Prahl, J., Deluca, H. F., and Denhardt, D. T. Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D_3 receptor and 1,25-dihydroxyvitamin D_3 enhancement of secreted phosphoprotein I (*Spp-1* or osteopontin) gene expression. Proc. Natl. Acad. Sci. USA, *87:* 9995–9999, 1990.