Okadaic Acid and Phorbol Esters: Comparative Effects of These Tumor Promoters on Cell Transformation, Intercellular Communication and Differentiation *in vitro*

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Okadaic acid is a non-12-O-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoter in the mouse skin carcinogenesis system. Here we report on the *in vitro* activity of okadaic acid in 3 assay systems: BALB/c 3T3 cell transformation, gap junctional intercellular communication (GJIC) in various cell types, and inhibition of induction of differentiation of Friend virus-transformed murine erythroleukemia (MEL) cells. The activity of okadaic acid was compared to that of the phorbol ester tumor promoters TPA and phorbol-12,13-didecanoate (PDD). In a test system involving a 2-week exposure of BALB/c 3T3 cells following 3-methylcholanthrene initiation, okadaic acid at a concentration of 10 ng/ml was equipotent to PDD as a promoter of cell transformation (4.9 and 3.7 foci/dish, respectively). Longer exposures to okadaic acid resulted in cytotoxicity. Okadaic acid-generated as well as PDD-generated transformed foci displayed a selective lack of GJIC between focus cells and surrounding normal cells, i.e., transformed cells communicate among themselves but not with surrounding cells. However, in contrast to TPA, there was no inhibition by okadaic acid, except at toxic doses, of homologous GJIC in BALB/c 3T3 cells or human and mouse keratinocytes. Furthermore, okadaic acid, unlike TPA, did not inhibit MEL cell differentiation. Together, these results indicate that okadaic acid acts as a promoter of cell transformation but that its mechanism of action is different from that of the phorbol ester tumor promoters.

Key words: Intercellular communication — *In vitro* cell transformation — Murine erythroleukemia cell differentiation — Okadaic acid — Phorbol ester

Studies to establish the phenomenology and to elucidate mechanisms of mouse skin tumor promotion have predominantly employed phorbol esters, and principally 12-O-tetradecanoylphorbol-13-acetate (TPA⁵).¹⁾ Recently though, the discovery of different classes of compounds active as promoters in mouse skin initiation/promotion assays has given credence to the idea that multiple mechanisms of tumorigenesis exist. For example, palytoxin,²⁾ thapsigargin³⁾ and chrysarobin⁴⁾ are non-TPA-type tumor promoters. In addition, okadaic acid was reported to be the most potent of the known non-TPA-type mouse skin tumor promoters.⁵⁾ Okadaic acid is a polyether compound present in certain sponges and shellfish species that are known to cause diarrhetic shellfish poisoning.⁶⁾

The mechanism of action of okadaic acid as a tumor promoter is different from that of TPA. In a competitive binding study, okadaic acid did not compete with TPA for specific binding to a mouse skin particulate fraction, nor did it activate protein kinase C (PKC), the proposed TPA receptor.⁵⁾ However, like TPA, okadaic acid is an irritant and an ornithine decarboxylase inducer in mouse skin.⁵⁾ In order to investigate further the biological activity of okadaic acid, we employed three different assay systems that are being used to detect tumor-promoting activity of chemicals and to study mechanisms of tumor promotion. Firstly, the BALB/c 3T3 cell transformation assay: designed to simulate *in vivo* carcinogenesis with its focus formation of tumorigenic, morphologically altered cells surrounded by non-transformed cells, this assay can be adapted for a 2-stage protocol of initiation/promotion⁷⁾; various *in vivo* tumor promoters including TPA and dihydroteleocidin B are active as promoters of this cell transformation.^{7,8)} Secondly, the inhibition of gap junctional intercellular communication (GJIC) assay: GJIC is the direct cell-cell exchange via gap junctions of ions and small molecules (<10³ daltons) and is considered essential for cell-cell cooperativity and tissue homeostasis.⁹⁾ Many tumor promoters including phorbol

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⁵ Abbreviations: TPA, 12-O-tetradecanoylphorbol-13-acetate; DMBA, 7,12-dimethylbenz[a]anthracene; FBS, fetal bovine serum; GJIC, gap junctional intercellular communication; MCA, 3-methylcholanthrene; MEL, murine erythroleukemia; MEM, minimal essential medium; NHEK, normal human epidermal keratinocytes; PDD, phorbol-12,13-didecanoate; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; TGF- β , transforming growth factor β .

esters inhibit GJIC and this has been proposed as a mechanism by which initiated cells could be divorced from gap junction-mediated growth-regulatory influences of surrounding normal cells.¹⁰⁻¹² Thirdly, the mouse erythroleukemia (MEL) cell differentiation inhibition assay: inhibition of cell differentiation has been proposed as one way in which tumor promoters could, in self-renewal systems such as hematopoietic and epithelial tissues, produce accumulation of otherwise-differentiating preneoplastic clones¹³; phorbol ester tumor promoters are potent inhibitors of MEL cell differentiation.¹⁴

The activity of okadaic acid was determined in these assay systems and compared to that of phorbol-12,13-didecanoate (PDD) in the BALB/c 3T3 cell transformation assay and that of TPA in the GJIC and differentiation assays.

MATERIALS AND METHODS

Chemicals Okadaic acid was isolated and purified from the black sponge *Halichondria okadai* as previously reported.¹⁵ TPA was obtained from CCR Inc., Eden Prairie, MN. All other chemicals were purchased from Sigma Chemical Co., St Louis, MO.

Cell culture BALB/c 3T3 cells (clone A31-1-1) were kindly provided by Dr. T. Kakunaga and were maintained in minimum essential medium (MEM) (Flow) containing 10% fetal bovine serum (FBS). Normal human epidermal keratinocytes (NHEK; secondary cultures), obtained after enzyme digestion of surgically discarded adult body skin, were provided by Prof. J. Thivolet (Hôpital Edouard Herriot, Lyon). SENCAR mouse primary keratinocyte cultures were prepared from the dorsal skin of 6- to 9-week-old female SENCAR mice (NCI, Frederick, MD), as previously described.¹⁶ The SENCAR mouse keratinocyte lines 3PC 1.1 and P1/17 FA were provided by Drs. R. Klann and T. J. Slaga (University of Texas System Cancer Center, Smithville, TX). P1/17 FA was derived from several pooled papillomas appearing on one SENCAR mouse 13 weeks into a 7,12-dimethylbenz[*a*]anthracene (DMBA)-TPA initiation-promotion protocol, while 3PC 1.1 was cloned from a parent line obtained after exposure of SENCAR primary keratinocytes to DMBA *in vitro* followed by selection in a high Ca^{2+} ($\approx 1.2 \text{ mM}$) medium.¹⁶ Keratinocytes were maintained in a low Ca^{2+} ($\approx 0.05 \text{ mM}$) MEM with 10% Ca^{2+} -free FBS.¹⁶ MEL cells were maintained in suspension in Eagle's basal medium supplemented with 10% FBS.¹⁷

In vitro cell transformation The BALB/c 3T3 cell transformation assay was carried out essentially as previously described.⁷ Briefly, 10^4 A31-1-1 cells were seeded into each 60 mm culture dish (Falcon #3002), and 24 h later, were exposed to $0.5 \mu\text{g/ml}$ of the initiator 3-methylcho-

lanthrene (MCA) for 72 h. The cells were rinsed then fed twice per week for 2 or 4 weeks with medium containing PDD, okadaic acid or solvent (control). Four weeks after initiation, the cultures were stained (Giemsa) and scored.

Tumorigenicity in nude mice Each clone of BALB/c 3T3 cell transformants, induced by MCA initiation and okadaic acid promotion, was suspended (10^6 cells) in 0.1 ml of MEM and injected subcutaneously into nude mice (Balb/c *nu/nu*; IFFA CREDO, L'Arbresle, France).

Measurement of GJIC GJIC was measured by means of the dye transfer method.¹⁸ Lucifer Yellow CH (10% w/v in 0.33 M LiCl) was injected into cells in monolayer culture with a vertical-type microinjection unit (Olympus IMT-2 SYF2, Tokyo) combined with a fluid pressure-manipulated X-Y stage (trial product, Narishige-Olympus) and gas pressure injector (#5242 Eppendorf, Hamburg). At 10 to 15 min after each injection, dye-coupled cells were scored by fluorescence detection with the same microscope.

MEL cell differentiation assay Two clones of MEL cells were used in this study: clone TS 19-101 with high sensitivity to TPA-mediated inhibition of differentiation and clone TR 19-9, resistant to TPA-mediated inhibition of cell differentiation.¹⁷ Differentiation to hemoglobin-producing cells was induced by addition of 4 mM hexamethylene bisacetamide; 4-5 days later, the percentage of hemoglobin-containing cells was determined by employing the benzidine reaction essentially as previously described.¹⁷

RESULTS

In vitro cell transformation The activities of okadaic acid and PDD in the BALB/c 3T3 cell transformation assay are shown in Fig. 1. In the absence of MCA initiation, a 2-week okadaic acid exposure (10 ng/ml) followed by 2 weeks without the compound elicited 0.7 foci/dish compared to 0.1 foci/dish for PDD. However, in a 4-week exposure, okadaic acid was cytotoxic whereas PDD alone generated numerous foci (5.2 per dish). For a 2-week-promoter/2-week-no-promoter protocol after MCA initiation, okadaic acid and PDD were equipotent with 4.9 and 3.7 foci/dish, respectively, at 10 ng/ml .

Following MCA and 4-week promoter treatment, PDD (10 ng/ml) yielded a large number of transformed colonies (24.8 foci/dish). With the same initiation/promotion protocol, okadaic acid, at the non-toxic 3 ng/ml dose, clearly promoted focus formation (1.8 foci/dish compared to 0.2 and 0 for the MCA and okadaic acid controls, respectively). However, at 10 ng/ml in this protocol, okadaic acid exerted such marked cytotoxicity and induced such extensive morphological changes that unequivocal scoring of transformed foci was difficult.

Fig. 1. Effects of okadaic acid (OA) and PDD on cell transformation of BALB/c 3T3 A31-1-1 cells. Cells with or without an MCA initiating dose were exposed to OA or PDD for 4 weeks or for 2 weeks with a 2-week recovery period. Data are presented as mean $\pm$ SD of 10–16 dishes/group. See "Materials and Methods" for details. * Density of surrounding cells was too low to score the foci.

Table I. Effects of Okadaic Acid (OA) and TPA on GJIC in Various Cell Types

Cells ^{a)}	Exposure time (h)	No. of dye-coupled cells/injection ^{a)}				Morphological changes ^{b)}		
		Control	TPA 100 ng/ml	OA 100 ng/ml	OA 10 ng/ml	TPA 100 ng/ml	OA 100 ng/ml	OA 10 ng/ml
1. NHEK	1	28.2 $\pm$ 7.1	16.3 $\pm$ 11.5	20.2 $\pm$ 6.6	27.0 $\pm$ 4.4			
	5	32.7 $\pm$ 9.7	11.0 $\pm$ 5.3	12.4 $\pm$ 3.8	25.4 $\pm$ 9.2	—	+	—
	29					+	det.	+
2. Mouse primary keratinocytes	1	30.5 $\pm$ 8.7	1.4 $\pm$ 1.6	11.6 $\pm$ 4.6	22.9 $\pm$ 8.7	+	+	—
	6.5					+	det.	$\pm$
3. 3PC 1.1	1	37.4 $\pm$ 13.2	38.4 $\pm$ 14.6	40.5 $\pm$ 11.1				
	7	39.3 $\pm$ 18.1	43.0 $\pm$ 17.0	16.6 $\pm$ 14.7		—	+	
	96					—	det.	
4. P1/17 FA	1	16.7 $\pm$ 6.4	2.5 $\pm$ 2.5	17.6 $\pm$ 5.5		—	—	
	3.5	13.3 $\pm$ 6.1	5.6 $\pm$ 4.2	9.8 $\pm$ 3.4		—	+	
5. BALB/c 3T3 A31-1-1	0.5	56.8 $\pm$ 12.6	3.2 $\pm$ 2.1	57.8 $\pm$ 15.8	55.4 $\pm$ 9.5	+	+	—
	4	54.4 $\pm$ 5.9	2.5 $\pm$ 1.4	14.0 $\pm$ 4.1	48.8 $\pm$ 15.1	+	det.	—

Confluent cultures were assayed for GJIC capacity as described in "Materials and Methods."

a) Data are mean $\pm$ SD of 15–30 separate injections.

b) Cells were noted as morphologically altered (+), unaltered (—), or detached from the dish (det.).

c) See "Materials and Methods" for a description of the cells.

Six MCA plus okadaic acid-induced foci (3 each for 2-week and 4-week okadaic acid treatment) were cloned and examined for tumorigenicity in nude mice; all 6 clones developed tumors within 2 weeks.

Effects on GJIC Table I presents the data on the effects of okadaic acid and TPA on GJIC in the human and mouse keratinocytes and the BALB/c 3T3 cells. TPA, at 100 ng/ml, inhibited within 1 h GJIC of all cell types except the TPA-resistant mouse keratinocyte line 3PC 1.1. For NHEK and the mouse papilloma line P1/17 FA, the TPA effect was not accompanied with morphological alterations. In contrast, okadaic acid at 100 ng/ml inhibited GJIC in all cell types but always elicited concomitant morphological changes (cells showed a globular shape and cell detachment was eventually observed for all types except P1/17 cells over the observation period). A 10 ng/ml dose of okadaic acid was also employed for NHEK, SENCAR primary keratinocytes and BALB/c 3T3 cells; again, the compound showed an inhibitory effect on GJIC only when morphological alterations occurred concomitantly. Almost invariably, exposure to okadaic acid for more than a few hours resulted in cell detachment.

To determine the long-term effect on GJIC of relatively low doses of okadaic acid, BALB/c 3T3 cells were

plated at 10^4 cells/dish, then exposed to 0, 1, 3 or 10 ng/ml okadaic acid for up to 18 days (medium changed twice per week). Cells were counted to establish growth curves while GJIC was studied from the time when the cells reached confluence. These data are presented in Fig. 2. Little effect was noted except for the 10 ng/ml dose. Here, although logarithmic growth was unaffected, the confluent cells appeared to be sensitive to the compound and cell density was decreased transiently to 15% of the control saturation density at day 13. GJIC paralleled this transient decrease. In order to determine whether the effect on GJIC was direct or occurred as a result of cell density decrease, the area of dye spread was examined closely at 6 days and 13 days after the start of 10 ng/ml okadaic acid exposure. At 13 days, as long as cells were in contact, the injected dye could spread an equal distance to that observed in the confluent cells at 6 days' exposure. Thus, the okadaic acid-induced decrease in GJIC at 13 days is not due to loss of intrinsic GJIC capacity but rather due to a decrease in cell number.

We have previously reported that, for transformed BALB/c 3T3 cell foci generated by various carcinogens and phorbol ester tumor promoters, there was a lack of GJIC between transformed cells and surrounding non-transformed cells, though transformed cells themselves

Fig. 2. Growth curves and GJIC capacity of BALB/c 3T3 cells grown in the presence of various concentrations of okadaic acid. ■, □: control; ◆, ◇: 1 ng/ml; ▲, △: 3 ng/ml; *, ×: 10 ng/ml.

Fig. 3. Phase contrast (upper panel) and fluorescence (lower panel) photographs of an MCA-okadaic acid-generated BALB/c 3T3 A31-1-1 transformed focus and surrounding normal cells after injection of Lucifer Yellow into a single normal cell (*). Note that dye spread is confined to the normal cells.

were GJIC-competent.¹⁹⁾ In this study, we examined 9 transformed foci *in situ* obtained after MCA initiation and okadaic acid promotion; all 9 foci displayed this lack of heterologous communication (Fig. 3).

Effect on MEL cell differentiation Fig. 4 shows a dose-response relationship for okadaic acid effects on MEL cell differentiation. Abrupt cytotoxicity was seen at doses above 3 ng/ml for both TPA-sensitive and TPA-resistant cells; this was associated with a complete lack of differentiation. Thus the lack of differentiation observed is accounted for by the cytotoxicity of okadaic acid to MEL cells. Therefore, these results give no evidence for a specific inhibitory effect of okadaic acid on MEL cell differentiation.

Fig. 4. Effect of okadaic acid on MEL cell growth and differentiation. A, TPA-sensitive 19-101 cell line; B, TPA-resistant 19-9 cell line. Cells were seeded at 10^5 /ml and exposed to HMBA and okadaic acid. Cell number and extent of differentiation were determined after 5 days.

DISCUSSION

The purpose of the present study was to compare the activity of tumor promoters, okadaic acid and phorbol esters, in three *in vitro* biological assays that we employ to detect the tumor-promoting activity of chemicals.

In all of the assays, okadaic acid displayed a greater toxicity than either TPA or PDD. This is reportedly true also in studies employing Swiss 3T3 cells and C3H10T1/2 cells,²⁰⁾ and therefore presents a problem in that prolonged culture is required for cell transformation of C3H10T1/2 cells²⁰⁾ and BALB/c 3T3 cells (this study). We found that, at 10 ng/ml, okadaic acid is not toxic during the growing phase of BALB/c 3T3 cells but it reduced the cell density several days after confluency. This extraordinary mode of toxic effect by okadaic acid suggests that this compound may modulate message(s) of growth arrest owing to contact inhibition; such contact inhibition is a property not retained by cancer cells. Since little is understood about contact inhibition of cell growth, it would be interesting to know the precise mechanism of this unusual mode of toxicity of okadaic acid. However, by limiting okadaic acid exposure in the

BALB/c 3T3 cell transformation assay to 2 weeks instead of the customary 4–5 weeks, 10 ng/ml okadaic acid was not detectably toxic and was found to be as potent a promoter as PDD. This then constitutes the first report that this non-TPA-type tumor promoter is active in an *in vitro* initiation/promotion cell transformation assay. A 4-week exposure to 3 ng/ml okadaic acid also resulted in clear promotion of transformation.

Previously, we have studied the generation of transformed BALB/c 3T3 cell colonies using UV, N-methyl-N'-nitroso-N-nitrosoguanidine, MCA or v-H-*ras* transfection, as well as these initiators combined with phorbol esters or transforming growth factor- β (TGF- β). In studying the GJIC characteristics of transformed cells vis-à-vis surrounding normal cells, we have found, without exception, a complete lack of heterologous communication, i.e., the transformed cells' GJIC compartment is completely separate from that of the non-transformed cells.^{19, 21, 22} Such is also the case here for MCA plus okadaic acid-generated transformed foci. It is possible that initiated cells are endowed with the property of selective GJIC by the carcinogen or oncogene but that this phenotype is not expressed unless exposure to a promoter ensues. Although the molecular mechanisms of such selective GJIC compartmentalization are not known, we recently found that both transformed cells and surrounding non-transformed cells express similar levels of mRNA of connexin 43, which is considered to be the major gap junction protein of BALB/c 3T3 cells.

In various studies, the ability of compounds, including phorbol ester derivatives, to inhibit GJIC has been found to correlate with tumor-promoting activity *in vivo*,²³ and this has led investigators to consider that inhibition of GJIC may be a critical action of tumor promoters in effecting escape of initiated cells from the growth confines of surrounding normal cells.^{10–12} There are, however, some exceptions; for example, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (*in vivo* promoter) and TGF- β promote *in vitro* cell transformation but do not inhibit GJIC.^{22, 24} In the present study, okadaic acid at non-toxic doses did not bring about any significant reduction in GJIC in a variety of cell types. It appears that okadaic acid enhances cell transformation without directly affecting GJIC and thus is different from other tumor-promoting agents, such as phorbol esters.

Phorbol ester tumor promoters inhibit various programs of cell differentiation in various cell types including MEL cells.¹³ Contrary to the case with TPA,¹⁷ inhibition of MEL cell differentiation by okadaic acid was seen only at cytotoxic doses; moreover, the effect of okadaic acid was very similar in a TPA-sensitive clone and a TPA-resistant clone, clearly showing that the two compounds are not interacting with the same biochemical pathways in the cells.

Clearly then, if effects on GJIC and/or differentiation are important for promoter activity of phorbol esters, other mechanisms of promotion must be envisaged for okadaic acid. Okadaic acid does not bind to the phorbol ester receptor or activate PKC,⁵ but the existence of a specific membrane receptor has recently been demonstrated.²⁵ Okadaic acid induces phosphorylation patterns very different from those induced by TPA²⁶ and, in comparison with TPA, induces modest levels of the so-called TPA-induced sequence genes in Swiss 3T3 cells, but with a delayed time response.²⁰ Since this induction appeared to be attenuated by a TPA-mediated down-regulation of PKC,²⁰ there is some hint that the PKC signalling pathway may play a role at least in okadaic acid induction of these genes. In addition, okadaic acid is a potent inhibitor of the serine/threonine-specific protein phosphatases,^{27, 28} suggesting further a link to signal transduction pathways which are mediated by serine/threonine-specific protein kinases such as PKC or cyclic AMP-dependent protein kinase (PKA). Since gap junction protein is likely to be a substrate of both PKC and PKA,^{29, 30} this latter result suggested to us that okadaic acid might exhibit a synergistic effect with TPA on GJIC inhibition; however, preliminary results with BALB/c 3T3 cells did not support this notion.

Recently, two derivatives of okadaic acid³¹ with different residues at the seventh carbon position were investigated, and okadaic acid and dinophysistoxin-1, which have a hydroxy group at C-7, had stronger activity than 7-O-palmitoyl okadaic acid in the mouse skin tumor promotion assay. Use of these analogues in our *in vitro* assays may be helpful in understanding structure-activity relationships.

In conclusion, we have reported here that the strong non-TPA-type tumor promoter, okadaic acid, is also a promoter of 2-stage cell transformation *in vitro* and that, unlike phorbol ester tumor promoters, its mechanism of action may not be linked to any disturbance of cell differentiation or gap junctional intercellular communication. Further elucidation of the mode of action of okadaic acid and that of other non-TPA-type promoters should improve our understanding of the multiple mechanisms of tumor promotion.

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