Phorbol Ester-mediated Inhibition of Intercellular Communication in BALB/c 3T3 Cells: Relationship to Enhancement of Cell Transformation

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ABSTRACT

Tumor-promoting phorbol esters reversibly inhibit intercellular communication between BALB/c 3T3 cells. In order to study the possible role of blocked intercellular communication in the promotion step in cell transformation, we investigated the effect of phorbol ester tumor promoters on cell transformation and intercellular communication in BALB/c 3T3 A31-1-1 cells by a dye-transfer method. When the cells are in the growing phase, inhibition of dye transfer by phorbol esters is complete but transient; more than 90% inhibition was observed 4 h after treatment of the cells with either 12-O-tetradecanoylphorbol-13-acetate or phorbol-12,13-didecanoate, but the extent of dye transfer returned to the control level after 24 h of treatment. However, when these phorbol ester-treated cells were cultured beyond confluence in the presence of tumor promoters, the capacity to transfer dye decreased again and was inhibited continuously for at least 5 weeks of culture. In control cultures, the extent of dye transfer between cells did not decrease at their confluence. The ability of 12-O-tetradecanoylphorbol-13-acetate and phorbol-12,13-didecanoate to induce continuous inhibition of dye transfer between these cells correlated well with their capacity to promote transformation of BALB/c 3T3 cells initiated with 20-methylcholanthrene. These results suggest that the continuously blocked intercellular communication after confluence, rather than its transient inhibition during the growing phase, might play an important role in the promotion of in-vitro two-stage transformation of BALB/c 3T3 cells.

INTRODUCTION

Phorbol esters, as represented by TPA,³ have been used widely as model compounds to study the mechanism of action of tumor promoters. Such studies have shown that these esters exert an extraordinarily wide range of biological and biochemical effects both in vivo and in vitro (1, 2). It is not clear which of their effects are essential to the mechanism by which they transform latent, initiated (presumably mutated) cells into malignant tumor cells, although it has been shown that they might act through their binding to protein kinase C (3–7). Considerable attention has been paid to their ability to inhibit gap-junctional intercellular communication, since this allows direct cell-to-cell transfer of compounds with low molecular weight and may therefore play a role in the control of cell proliferation and differentiation (8–10). Evidence that tumor promoters can inhibit cell-cell communication was first obtained independently by Yotti et al. (11) and Murray and Fitzgerald (12). Since that time, phorbol ester-mediated inhibition of cell communication has been observed and confirmed in many types of cultured cells, including human cells by means of metabolic cooperation (13, 14), electrical coupling (15, 16), and dye transfer methods (17–19). It has been shown that other types of possible promoting agents, such as phenobarbital, saccharin, and cigarette smoke condensates, inhibit intercellular communication between cultured cells (20, 21). Moreover, Yancey et al. (22) and Kalimi and Sirsat (23) have reported a decreased number of gap junctions in TPA-treated cultured cells and mouse skin, respectively. These observations suggest that tumor promoter-mediated inhibition of cell communication might play an important role in the clonal growth of initiated cells beyond normal growth limitation and/or in the final expression of pre- or malignant phenotype. However, it has also been observed that the inhibitory effect is transient in some types of cells; the effect was recovered completely within 12 h in C3H 10T½ cells (24), within 18 h in an established epidermal cell line (18), within 12 h in V79 cells (25), and within 24 h in cultured human cells (13). These results cast doubt on the hypothesis that tumor promoter-mediated inhibition of intercellular communication has an important role in the process of 2-stage (initiation-promotion) carcinogenesis in vivo and in vitro, since the production by tumor promoters of tumors in vivo or of transformed foci in vitro requires long-term and frequent application.

Recently, Dorman et al. (24) studied the possible role of blocked intercellular communication in in-vitro transformation of C3H 10T½ cells. They found no correlation between the dose of TPA required for promotion and the degree of inhibition of intercellular communication, and they found that inhibition of intercellular communication is transient. They concluded that inhibition of intercellular communication is not a sufficient event for promotion of transformation in these cells. However, they used the metabolic cooperation method to determine intercellular communication; with that method, it is not possible to measure the intrinsic intercellular communication capacity of cells that are in a state similar to that in which cell transformation occurs, since this assay requires replating of cells. Transformed foci become visible only after 3 to 5 weeks in in-vitro 2-stage transformation assays using BALB/c 3T3 and C3H 10T½ cells (26–28), and the metabolic cooperation assay does not permit examination of intercellular communication capacity during this process.

In our study to examine the possible relationship of phorbol ester-mediated inhibition of intercellular communication and their promoting ability in BALB/c 3T3 cell transformation, we used a dye transfer method, which made it possible to investigate intercellular communication occasionally during the whole period of in-vitro cell transformation. We report here the results of our investigations.
MATERIALS AND METHODS

Materials. Lucifer Yellow CH and MCA were purchased from Sigma Chemical Co., St Louis, MO. TPA and PDD were obtained from Chemicals for Cancer Research, Inc., Eden Prairie, MN. Capillary tubes used for microinjection were obtained from A-M Systems, Inc., Everett, WA. Cell Culture and in-Vitro 2-Stage Cell Transformation. BALB/c 3T3 A31-1-1 cells, obtained from Dr. T. Kakunaga, were cultured in Eagle’s minimal essential medium supplemented with 10% fetal calf serum. For induction of transformed foci, 1 \times 10^4 cells were seeded onto 60-mm Falcon plastic dishes, and MCA (0.1 \mu g/ml) was added to each culture dish on the following day. After 48 h of treatment, MCA was removed by washing with fresh medium and by replacement with promoter-containing medium. The culture medium was changed every 3 or 4 days for 5 weeks. Types of transformed focus were determined under a dissecting microscope after fixation and Giemsa staining. Only densely stained foci with clear criss-crossing of the cells at their periphery were scored as transformed foci (26, 28). Our preliminary data indicate that about 60% of these foci produce tumors when 1 \times 10^6 cells are inoculated into a nude mouse.

Measurement of Intercellular Communication by Means of Fluorescent Dye Transfer. Intercellular communication was measured by the dye transfer method, as described previously (17). Briefly, intercellular transfer of fluorescent Lucifer Yellow CH was measured by direct microinjection of the dye into a cell under a phase-contrast microscope and observation of its transfer to neighboring cells under a fluorescein phase-contrast microscope. Since this dye cannot diffuse through the membrane, its transfer into neighboring cells is believed to occur by gap-junctional communication (29-31). Microinjection was carried out using an Olympus Injectoscope (17). A 10% (w/v) solution of Lucifer Yellow CH in 0.33 M lithium chloride solution was transferred to a glass capillary needle, which was prepared from a capillary tube using an automatic magnetic puller (Narishige Co., Tokyo, Japan). A capillary needle containing Lucifer Yellow CH solution was attached to the Injectoscope; the cells were impaled with a needle; and the solution was injected by hand pressure for 3 to 5 s at room temperature.

The extent of dye transfer was measured at room temperature under a fluorescence phase-contrast microscope by scoring the number of fluorescent cells. Dye was transferred rapidly in control cultures. Transfer reached a plateau 5 min after injection, and this value was maintained for at least another 10 min (17). Measurement of dye transfer was therefore carried out 10 min after injection. Since it was found that functional dye transfer capacity is constant for at least 40 min after the cells are transferred to room temperature, microinjection of dye and observation of the extent of dye transfer were performed within 30 min after the cultures had been brought to room temperature (17). The results presented in each chart are taken from a series of experiments. Similar results were obtained in at least one other independent series of experiments.

RESULTS

Long-Term Effect of Phorbol Esters on Dye Transfer between BALB/c 3T3 Cells at Their Growing and Resting Phases. In order to determine the effect of tumor-promoting agents on intercellular communication during promotion of cell transformation, BALB/c 3T3 cells were cultured under similar conditions to those used for the cell transformation assay; 1 \times 10^4 cells/Petri dish were seeded on Day 0, and phorbol esters (TPA or PDD) were added on Day 3. Dye transfer was assayed from Day 5 after the cells were seeded, because the cell density prior to Day 5 was too low to assay the extent of dye transfer between cells quantitatively. On Day 5, the number of cells/dish in the control culture had reached 1.4 \times 10^6, and most cells were in contact. The growth rate began to decrease slightly thereafter, but most cells could divide another one or 2 times, as shown in Chart 1A. This slight cell growth continued up to Day 8; thereafter, the number of cells/dish began to decrease and reached a steady state on Day 13 of culture, which was maintained for at least another 3 weeks. In cultures treated with 100 ng of TPA or PDD per ml, exponential cell growth continued for 6 days after culture; thereafter, the growth curve was similar to that of the control culture, but the final saturation density of cells/dish was about 1.7-fold higher than that of the control culture. There was no significant difference in saturation density between TPA- and PDD-treated cultures (Chart 1A).

In the control culture, the number of dye-coupled cells/injection was 23.1 ± 6.7, 28.8 ± 4.6, and 29.8 ± 3.9 (SD) 5, 6, and 7 days after culture, respectively (Chart 1B; Fig. 1, A and B). Thereafter, the extent of dye transfer decreased slightly but had recovered to 31.1 ± 5.2 by Day 14 after culture; this level was maintained up to termination at 5 weeks of culture (Chart 1),

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indicating that the communicating capacity of nontreated BALB/c 3T3 A31-1-1 cells does not change during 5 weeks of culture.

In cultures treated with TPA or PDD (100 ng/ml) from Day 3, the extent of dye transfer was similar to that of the control culture until Day 7. Dye transfer capacity in promoter-treated cultures (Chart 1B) started to decrease from Day 8, when complete cessation of cell growth occurred; the numbers of dye-coupled cells/injection in TPA-treated cultures were 15.8 ± 6.6 and 6.5 ± 5.6 on Days 8 and 10, respectively, slightly increased up to 12.8 ± 6.2 on Day 14. This inhibition was maintained for at least another 3 weeks (Chart 1B; Fig. 2, C to F). The time course of the effect of PDD on dye transfer was basically the same as that of TPA, but its inhibitory effect was much greater; the number of dye-coupled cells were 1.7 ± 0.9, 2.1 ± 1.2, and 3.8 ± 3.2 on Days 9, 10, and 14 after culture, indicating that phorbol ester tumor promoters induce continuous inhibition of cell communication after confluence, but not during the growth phase.

Transient Recovery of Intercellular Communication from Phorbol Ester Effect. In order to further investigate the growth dependence of promoter-mediated inhibition of dye transfer, cells were treated with TPA or PDD (100 ng/ml) on Day 5 after culture, when dye transfer can be assayed quantitatively and can therefore be investigated at various intervals after exposure to promoters. The results are illustrated in Chart 2. When TPA or PDD (100 ng/ml) was added to the culture on day 5, a rapid decrease in dye transfer capacity was observed (Fig. 1, C and D); thereafter, the extent of dye transfer increased gradually and was completely restored 24 h later (Fig. 1, E and F), indicating that this rapid inhibition of dye transfer by promoters is transient. Most cells that are exposed to phorbol esters recover their dye-transfer capacity within a short time (<24 h), even in the presence of promoters. This recovery is not due to exhaustion of phorbol ester in the medium, since repeated additions of phorbol esters had no effect on the restoration of dye transfer (data not shown).

The restored capacity to transfer dye was maintained up to Day 7; however, the extent of dye transfer began to decrease again from Day 8 in promoter-treated cultures (Chart 2), and this decrease was not reversed up to termination of culture in the presence of promoters.

A similar pattern of inhibition was observed when PDD was added to confluent cultures of BALB/c 3T3 cells (Chart 2). Thus, when cells in the steady-state growth phase (Day 21 after culture) were treated with PDD, complete inhibition of dye transfer was observed 1 h later; however, the inhibition was transient, and dye-transfer capacity was completely restored 24 h later and maintained for at least 24 h. When these cells were further cultured in the presence of PDD, however, dye transfer capacity again decreased 7 days later. The transient recovery time from the effect of PDD coincides with that of cell growth, presumably due to the mitogenic effect of PDD, since when the cells reached their new saturation density (Chart 1A), inhibition was stabilized (Chart 2). These results indicate that the response of BALB/c 3T3 cells to phorbol esters occurs in 2 distinct phases in terms of inhibition of cell communication; the first phase is a rapid and transient inhibition of dye transfer during the growth phase, and the second phase is a relatively slow and continuous inhibition at complete confluence.

Relationship between Inhibitory Effect of Phorbol Esters on Dye Transfer and Their Ability to Promote BALB/c 3T3 Cell Transformation. The extent of continuous inhibition of dye transfer by phorbol esters was compared to their capacity to promote cell transformation in vitro as a function of dose. The enhancement of MCA-induced cell transformation by different doses of TPA and PDD is summarized in Table 1. A single treatment with MCA alone at 0.1 µg/ml induced 0.2 transformed focus per dish, whereas no spontaneous transformation occurred in untreated cultures. TPA or PDD alone induced transformed foci in a dose-dependent manner, indicating that non-MCA-treated BALB/c 3T3 cells might contain some already
intercellular communication and cell transformation

Table 1

<table>
<thead>
<tr>
<th>MCA initiation (0.1 μg/ml)</th>
<th>Promoter (ng/ml)</th>
<th>No. of transformed foci/10 dishes</th>
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<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
</tr>
<tr>
<td>- (Acetone)</td>
<td>0 (0.0)*</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>+ (Acetone)</td>
<td>2 (0.2)</td>
<td>2 (0.2)</td>
</tr>
<tr>
<td>TPA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>1.0</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>+</td>
<td>1.0</td>
<td>3 (0.3)</td>
</tr>
<tr>
<td>-</td>
<td>10.0</td>
<td>3 (0.3)</td>
</tr>
<tr>
<td>+</td>
<td>10.0</td>
<td>3 (0.9)</td>
</tr>
<tr>
<td>-</td>
<td>100.0</td>
<td>5 (0.5)</td>
</tr>
<tr>
<td>+</td>
<td>100.0</td>
<td>22 (2.2)</td>
</tr>
<tr>
<td>PDD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>1.0</td>
<td>2 (0.2)</td>
</tr>
<tr>
<td>+</td>
<td>1.0</td>
<td>9 (0.9)</td>
</tr>
<tr>
<td>-</td>
<td>10.0</td>
<td>23 (2.3)</td>
</tr>
<tr>
<td>+</td>
<td>10.0</td>
<td>95 (9.5)</td>
</tr>
<tr>
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<td>100.0</td>
<td>45 (4.5)</td>
</tr>
<tr>
<td>+</td>
<td>100.0</td>
<td>130 (13.0)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses, number of transformed foci per dish.

initiated or pretransformed cells. In cultures treated with MCA (0.1 μg/ml), TPA induced 0.3, 0.95, and 2.1 transformed foci/Petri dish at 1-, 10-, and 100-ng/ml doses, respectively; and PDD induced 1.05, 10.0, and 12.7 transformed foci/Petri dish at doses of 1.0, 10, and 100 ng/ml, respectively. Thus, the ability of PDD to induce cell transformation was much greater than that of TPA.

Inhibition of dye transfer in comparison to enhancement of transformation by different doses of TPA and PDD is shown in Chart 3. A dose-related inhibition of intercellular communication was observed with both compounds. Both short-term (4 h) and long-term (21 days) effects of TPA and PDD on intercellular communication were studied. Although the short-term effect of the 2 was very similar, PDD had a greater long-term effect than TPA; this is consistent with the results shown in Charts 1B and 2. Since PDD is more potent than TPA in enhancing cell transformation (Table 1; Chart 3), the long-term effect of these tumor promoters on intercellular communication correlates well with their ability to enhance cell transformation.

**DISCUSSION**

We have reported here that intercellular transfer of microinjected Lucifer Yellow CH between cultured BALB/c 3T3 cells is continuously inhibited by phorbol ester tumor promoters in a dose-dependent manner up to termination of culture after cells reach confluence. This continuous inhibition was observed only after transient recovery from the effects of phorbol esters. We have shown that the transient recovery of intercellular communication is accompanied by cell growth, and that growth arrest is essential to obtain continuous inhibition of intercellular communication. It was noted in previous studies that phorbol ester mediated inhibition of intercellular communication is transient (see “Introduction”). To our knowledge, this is the first study in which continuous inhibition of intercellular communication by phorbol ester tumor promoters for a long period has been shown, and we conclude that the effects of phorbol esters on intercellular communication are highly dependent on the growing phase of the target cells.

PDD was more effective than TPA in enhancing cell transformation and in blocking intercellular communication of BALB/c 3T3 cells, suggesting a good correlation between the ability of these compounds to promote cell transformation and their ability to inhibit intercellular communication. There was also a good correlation in the dose response of these 2 phorbol esters in promoting cell transformation and in long-term inhibition of intercellular communication. On the other hand, short-term effects of these compounds did not correlate well with their enhancing effects on cell transformation. These results suggest that blockage of intercellular communication may be involved in the mechanism by which tumor-promoting phorbol esters enhance cell transformation.

These results appear to be in contrast to those of Dormán et al. (24), who reported that the dose response of TPA in promoting transformation does not correlate with that for reducing cell communication and that TPA-mediated inhibition is transient in C3H 10T½ cells. However, in their experiments, cell were exposed to TPA for only 72 h, followed by trypsinization of preexposed cells before assay for metabolic cooperation. Trypsinization must induce many cells to go into the growth phase, and
we have shown in the present study that neither the short-term effects of TPA and PDD nor their effect on growing cells correlates with their enhancement of cell transformation. The finding that the short-term effect of TPA and PDD on intercellular communication does not correlate well with their enhancing effect on cell transformation is consistent with the notion that long-term treatment of carcinogen-treated cells with phorbol esters is a prerequisite for maximal enhancement of cell transformation.

Phorbol esters are thus maximally effective when their target cells are in confluence. A related phenomenon that we have found recently is a dramatic difference in the intrinsic capacity for intercellular communication between transformation-sensitive and transformation-resistant clones of BALB/c 3T3 cells (32, 33). Although these clonal cells have a similar communication capacity when in the growth phase, there was a remarkable decrease in intercellular communication, continuing for 4 weeks, only in sensitive clone cells at confluence, indicating that the loss of cell communication correlates well with cell transformation sensitivity (32, 33). These data reinforce the importance of blocked cell communication after confluence in the process of in-vitro cell transformation. Although the biochemical mechanisms by which the cells lose their intercellular communication capacity at confluence are not clear, our preliminary results indicate that a change in the pattern of the extracellular matrix, e.g., fibronectin, may be involved.

We also observed in this study that TPA has a much lower ability to decrease intercellular communication and to enhance cell communication than has PDD, although TPA enhances the growth of BALB/c 3T3 cells to the same extent as PDD. Hirakawa and Kakunaga (34) reported that PDD is more effective than TPA in enhancing the incidence of transformed foci, although there is no difference between TPA- and PDD-treated cultures in terms of total DNA content. They also showed a correlation between the rate of metabolism of these phorbol esters and their promoting ability in BALB/c 3T3 cells. We observed the same correlation. Part of our results may thus be explained by differences in the capacity of BALB/c 3T3 cells to metabolize phorbol esters.

Although our results suggest that blocked intercellular communication is involved in the promotion phase of cell transformation, the mechanism involved is not clear. We reported recently that cells in transformed foci do not communicate with surrounding nontransformed cells, although both types of cells can communicate with neighboring homologous cells, as revealed by the dye transfer method (26, 34). It is therefore likely that, in the absence of tumor-promoting agents, carcinogen-induced initiated or premalignant cells are inhibited in expressing their transformed phenotypes because they have harmonious intercellular communication with surrounding nontransformed cells; however, application of tumor promoters to such cultures promotes the final expression of transformed phenotypes of initiated or premalignant cells by blocking intercellular communication of these altered cells with surrounding normal cells.

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Fig. 1. Pattern of dye transfer in control (0.1% acetone) and TPA-treated cells during growth phase. Cells were cultured as described in the legend to Chart 1. TPA was added to the cultures on Day 5; 4 and 24 h after addition of TPA, the cells were injected with Lucifer Yellow CH (*) and photographed 10 min later. A, fluorescence photomicrograph of cells cultured in 0.1% acetone for 4 h; B, phase-contrast micrograph of A; C, cells cultured with TPA (100 ng/ml) for 4 h; D, phase-contrast micrograph of C; E, cells cultured with TPA for 24 h; F, phase-contrast micrograph of E.
Fig. 2. Pattern of dye transfer in cells cultured with TPA or PDD for 3 weeks. On Day 21 after culture with TPA or PDD (100 ng/ml), cells were injected with Lucifer Yellow CH (**) and photographed 10 min later. A, fluorescence photomicrograph of control cells 3 weeks after culture; B, phase-contrast micrograph of A; C, cells cultured with TPA for 3 weeks; D, phase-contrast micrograph of C; E, cells cultured with TPA for 3 weeks; F, phase-contrast micrograph of E.
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