Phorbol Ester Enhancement of IL-3-Dependent Proliferation of Primitive Hematopoietic Progenitors of Mice in Culture

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ABSTRACT

Protein kinase C (PKC) is a Ca$$^{2+}$$- and phospholipid-dependent protein kinase activated by diacylglycerol that is either released from cell membranes in response to certain growth factors or mimicked by 12-O-tetradecanoyl phorbol-13-acetate (TPA). We studied the effects of TPA on interleukin-3 (IL-3)-dependent colony formation of mouse bone marrow cells from mice injected with 5-fluorouracil 2 days before examination in order to clarify the significance of PKC in the proliferation of primitive hematopoietic progenitors. Although TPA alone did not support colony formation, TPA in combination with IL-3 increased colony numbers from 1.5 to 2 times that formed with IL-3 and vehicle. TPA increased not only the granulocyte/macrophage colonies, but also the multilineage colonies. A sequential colony count showed that TPA, unlike IL-6, did not hasten the appearance of colonies. Because TPA enhanced IL-3-dependent colony formation derived from lineage-negative marrow cells obtained from mice that received 5-FU 2 days before, it is possible that it might act directly on primitive progenitors. Prolonged pretreatment of marrow cells with TPA prevented TPA-augmented colony growth. Calphostin C, a specific PKC inhibitor, and certain specific tyrosine kinase inhibitors, such as genistein and herbimycin A, abrogated the enhancing effects of TPA on IL-3-dependent colony formation. These data suggest that TPA had a direct effect on the primitive progenitors and enhanced IL-3-dependent colony formation via activation of PKC and certain tyrosine kinases.

IL-3 is a multilineage hematopoietic cytokine that can support the survival and proliferation of immature multipotential progenitors and cells committed to a number of myeloid lineages (Ihle, 1992). IL-3 also supports the proliferation of factor-dependent cell lines such as FDCP-1 (Dexter et al., 1980) and B6SUtA1 (Sorensen et al., 1989). The receptor for human IL-3 consists of an α-subunit and a β-subunit, but both lack detectable catalytic domains (Ihle, 1992). Recent studies on postreceptor cytokine signaling have revealed that the binding of IL-3 to its receptor molecules results in their dimerization, thus activating two signaling pathways: a ras-mediated (ras-raf-1-MAP kinase) pathway (Satoh et al., 1992; Vojtek et al., 1993; Kyriakis et al., 1992) and a ras-independent JAK (Janus kinase)-STAT (signal transducer and activator of transcription) system (Silvennoinen et al., 1993; Ihle et al., 1994).

PKC (Nishizuka, 1984; 1988; 1992) has been reported to play an important role in the proliferation of IL-3-dependent cell lines. IL-3 stimulation of FDCP-1 cells has been reported to induce the translocation of PKC from cytosol to membrane (Farrar et al., 1985). Although this observation has been confirmed in some studies (Whetton et al., 1986; Pelech et al., 1990), it has not been confirmed in others (Ihle, 1992). Recently, it has been shown that PKC activates Raf-1 by direct phosphorylation in NIH 3T3 fibroblasts (Kolch et al., 1993, Burgering and Bos, 1995). These findings are very important in demonstrating the cross-talk of PKC activation and ras-mediated IL-3 signal transduction pathways. However, it is not certain that these observations are applicable to normal hematopoiesis.

In the presence of IL-3, certain cytokines, such as IL-6 (Ikebuchi et al., 1987), granulocyte colony-stimulating factor (G-CSF; Ikebuchi et al., 1988), IL-11 (Musashi et al., 1991a), etc., enhance IL-3-induced primitive hematopoiesis.

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ABBREVIATIONS: Day-2 post 5-FU marrow cells, bone marrow cells obtained from mice that received i.v. 5-FU 2 days before; DMSO, dimethyl sulfoxide; Ep, erythropoietin; GM, granulocyte/macrophage; GEMM, granulocyte erythrocyte/macrophage/megakaryocyte; 5-FU, 5-fluorouracil; IC$$^{50}$$, 50% inhibition constant; IL-3, interleukin-3; OAG, 1-oleoyl-2-acetyl-glycerol; PKC, protein kinase C; TPA, 12-O-tetradecanoyl phorbol-13-acetate.
c-kit ligand (Tsujii et al., 1991) and IL-12 (Jacobson et al., 1993; Hirayama et al., 1994), have been observed to be capable of exerting synergistic effects on the proliferation of primitive hematopoietic progenitors. These particular cytokines are termed “synergistic factors” (Ogawa, 1993). On the basis of these studies, it has been suggested that IL-3 can support the proliferation of progenitors that have left the dormant state of the cell cycle (G0) but that it is unable to trigger the recruitment of primitive progenitors into the cell cycle (Suda et al., 1985; Leary et al., 1989). On the other hand, these synergistic factors augment IL-3-dependent colony growth by shortening the G0 period (Ikebuchi et al., 1987, 1988; Musashi et al., 1991a; Tsujii et al., 1991; Hirayama et al., 1994). Interestingly, in addition to its synergistic effects on IL-3-dependent colony formation, c-kit ligand stimulates the proliferation of progenitors that have left the G0 state, so it is able to exert synergistic effects on other “synergistic factors” (Tsujii et al., 1991). Thus the synergistic interaction of these cytokines is somewhat complicated, and it is important that their signal transduction pathways, along which cellular responses will be made, be clarified.

As a first step, we studied the PKC activator TPA (Niedell et al., 1983) in order to determine whether it could enhance the IL-3-dependent proliferation of primitive hematopoietic progenitors in mice, in an effort to clarify the significance of PKC in the proliferation of primitive hematopoietic progenitors.

Materials and Methods

Cell preparation. Ten to 15-week-old male BDF1 ((C57 B1/6 × DBA/2 F1 hybrids) mice were obtained from Charles River Japan (Atsugi, Japan). A single-cell suspension was prepared from the pooled femurs of the mice, which had been injected with 150 mg/kg b. w. of 5-FU (Kyowa Hakko Kogyo Co., Tokyo, Japan) i.v. through their tail veins 2 days before examination (Day-2 post 5-FU marrow cells) in order to enrich the noncycling hematopoietic primitive progenitors (Hodgson and Bradley, 1979; Suda et al., 1983). Lineage-negative (Lin−) Day-2 post 5-FU marrow cells were isolated as described by Shih et al. (1992) with some minor modifications. Briefly, light-density cells were separated by density centrifugation above Ficoll-Conray (specific gravity, 1.077) from the Day-2 post 5-FU marrow cells. They were then incubated at 4°C for 45 min in a cocktail of antibodies: anti-Thy 1.2 (Pharmingen, San Diego, CA), B220 (CD45R, Pharmingen), Gr-1 (Pharmingen), and Mac-1 (CD11b, Boehringer Mannheim Biochemica, Germany). After washing twice, sheep anti-rat IgG (Fc)-conjugated immunomagnetic beads (Dinabeads, Dynal A.S., Oslo, Norway) were added to the cell suspension and incubated at 4°C for 45 min. Lineage-specific-antigen-positive (Lin+) cells were removed by a magnetic particle concentrator (Dynal), and Lin− cells were recovered from the supernatant. The cell/bead ratio applied was 1:30.

Factors and agents. The source of recombinant murine IL-3 was medium conditioned by Chinese hamster ovary (CHO) cells that had been genetically engineered to produce murine IL-3 to high titer (approximately 70,000 U/ml). Human recombinant Ep was a generous gift from Kirin Brewers Co. (Tokyo, Japan). TPA and OAG were purchased from Sigma Chemical Co. (St. Louis, MO). Calphostin C, a specific inhibitor of PKC, was purchased from Kyowa Medica Co. (Tokyo, Japan). The IC50 values of calphostin C against PKC, cyclic AMP-dependent protein kinase (A-kinase) and tyrosine kinase (p60src) were reported to be 0.05 μM, >50 μM and >50 μM, respectively (Kobayashi et al., 1989a, b).

Genistein (Akiyama et al., 1987) and herbimycin A (Uehara et al., 1988; Uehara et al., 1989) were used as the tyrosine kinase inhibitors. Genistein was purchased from Sigma. The IC50 values of genistein have been reported to be 8 μg/ml (p60src), 6 μg/ml (epidermal growth factor receptor) and >100 μg/ml (PKC, A-kinase) (Akiyama et al., 1987). The IC50 values of herbimycin A have been reported to be 5 μM (tyrosine kinase, p210mer/abl), 12 μM (tyrosine kinase, p60src), >350 μM (PKC) and >350 μM (A-kinase) (Fukazawa et al., 1991). All the agents were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the culture dishes was less than 0.1%.

Clonal cell culture. Methyllumulose cell culture was performed in 35-mm Lux suspension culture dishes (No. 5221R, Nunc Inc., Naperville, IL) as described previously (Musashi et al., 1991a, b). One milliliter of culture contained 5 × 104 Day-2 post 5-FU marrow cells or 2000 lineage-negative cells, alpha-medium (Flow Laboratories Inc., Rockville, MD), 1.2% methylcellulose (Wako Junyaku Co., Tokyo, Japan), 30% fetal calf serum (HyClone Laboratories, Logan, UT), 1% fraction V bovine serum albumin (Sigma), 2 μM of recombinant human Ep, 100 μM 2-mercaptoethanol (2-ME, Eastman Kodak, Rochester, NY), hematopoietic factors and agents. The dishes were incubated in a humidified atmosphere flushed with 5% CO2 at 37°C. Colonies consisting of 50 or more cells were counted on indicated days under an inverted microscope according to colony types (Nakahata et al., 1982) as indicated in the tables. Briefly, GM colonies consisted of large, round macrophages and polygonal neutrophils. GEMM colonies were recognized by the red or dark brown color of hemoglobin in aggregated erythrocytes and by huge megakaryocytes adding to granulocytes and macrophages. The blast cell colonies consisted of a homogeneous population of up to 1000 loosely arranged, frequently clumped, round cells with no signs of terminal differentiation such as granulocytes, macrophages, erythrocytes, and megakaryocytes. The blast cell colonies would later develop into GEMM colonies or large GM colonies.

For OAG supplementation assay, on day 2 of the culture, a supplement of OAG was added by layering 0.1 ml of 10−6 M OAG over the surface of each 0.9-ml culture containing 5 × 104 Day-2 post 5-FU marrow cells, 100 U of IL-3 and 10−7 M OAG.

Protein kinase inhibitors were added to the cell suspensions at least 10 min before the addition of TPA or IL-3.

Unless otherwise stated, data represent mean ± S.D. from quadruplicate dishes.

Effects of long-term preincubation of cells with TPA on colony growth. Long-term incubation of cells with TPA resulted in a down-regulation of PKC (Kitajima et al., 1988; Goodnight et al., 1994). To examine whether TPA exerts its effects through the activation of PKC, one million Day-2 post 5-FU marrow cells were incubated in alpha-medium with 2 × 10−8 M TPA or vehicle for 48 hr. Neither the FCS nor the cytokines were supplemented in the cell suspension. After washing, the cells were incubated in a methylcellulose culture containing IL-3 either alone or in combination with TPA.

Preincubation of cells. To infer the signal transduction of TPA by using the protein kinase inhibitors, Day-2 post 5-FU bone marrow cells were preincubated for 12 hr with TPA either alone or in combination with the inhibitors. After washing twice, the cells were cultured in methylcellulose containing IL-3.

Statistical analysis. Student’s t test was used for the statistical analysis. For analysis of variance (ANOVA), an F test was performed on the data before Student’s t test.

Results

Colony formation derived from Day-2 post 5-FU marrow cells. We first analyzed the colony formation derived from Day-2 post 5-FU marrow cells supported by IL-3 in combination with TPA. Whereas TPA alone did not stimulate colony growth, it did augment colony formation when in combination with IL-3, maximally at 10−7 M. This particular
concentration of TPA, in combination with IL-3, increased colony growth by 175% ± 16% (mean ± S.D.) in nine of the experiments compared with the IL-3 plus vehicle control. Table 1 shows a representative result. A higher concentration of TPA (10^{-6} M) seemed to suppress colony growth. The final concentration of DMSO that contained 10^{-7} M TPA was 0.01%. Because concentrations of DMSO less than 0.1% did not affect IL-3-dependent colony formation (data not shown), this suppressive effect of TPA is not due to DMSO. Another PKC activator, OAG, at a concentration of 10^{-6} M and 10^{-7} M also, significantly enhanced IL-3-dependent colony formation. However, the degree of increase was less than that for those augmented with TPA. The addition of supplement OAG (final concentration, 10^{-7} M) on the second day to those cultures containing 10^{-7} M OAG resulted in an increase in colony numbers (19 ± 2) compared with those supplemented with vehicle (16 ± 2) and those that had not been supplemented (15 ± 2). These data suggest that OAG may be inactivated rapidly in the culture dishes.

TPA increased not only the GM colonies but also the GEMM colonies (Table 2).

**Serial observation of blast cell development in Day-2 post 5-FU marrow cells.** To examine the time course of colony development, we observed the culture dishes every third day for a serial observation of blast cell development. As shown in figure 1, although TPA augmented IL-3-dependent colony growth, unlike IL-6, it did not hasten the appearance of colonies supported by IL-3. The addition of TPA did not affect the synergistic effects of IL-3 + IL-6.

To analyze further the kinetics of colonies supported by IL-3 + TPA, we examined the culture dishes daily and recorded the development of new blast cell colonies and their subsequent proliferation in cultures containing IL-3 + vehicle and IL-3 + TPA (“mapping studies,” fig. 2). On the basis of the number of days required for colonies to reach 100 cells was calculated to be 10.8 ± 2.9 and 10.2 ± 2.3 in cultures supported by IL-3 + vehicle and by IL-3 + TPA, respectively. The average number of days required for colonies to reach 100 cells was calculated to be 17.5 ± 4.3 hr and 17.3 ± 2.9 hr in cultures supported by IL-3 + vehicle and by IL-3 + TPA, respectively. The average number of colonies found to be smaller than 150 cells per colony as stimulated by IL-3 on day 9 of the culture. After washing, the pooled blast cells were reincubated with IL-3 or IL-3 + TPA in cultures containing 30% fetal calf serum, 1% bovine serum albumin, and 2 U/ml of Ep. Both IL-3 + TPA and IL-3 + vehicle stimulated secondary colony formation in the pooled blast cells, and the plating efficiencies were 15% to 20% and 8% to 12%, respectively (table 3). These data suggested that TPA had, to some extent at least, acted on the early progenitors directly.

### Table 1

**Effects of TPA on colony formation in Day-2 post 5-FU marrow cells**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Number of Colonies</th>
<th>Day 8</th>
<th>Day 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-3</td>
<td>4 ± 1</td>
<td>14 ± 2</td>
<td>2</td>
</tr>
<tr>
<td>IL-3 + vehicle</td>
<td>4 ± 2</td>
<td>13 ± 2</td>
<td>2</td>
</tr>
<tr>
<td>IL-3 + TPA</td>
<td>10^{-6} M</td>
<td>5 ± 2</td>
<td>18 ± 2*</td>
</tr>
<tr>
<td>IL-3 + TPA</td>
<td>10^{-7} M</td>
<td>6 ± 2</td>
<td>24 ± 1**</td>
</tr>
<tr>
<td>IL-3 + OAG</td>
<td>10^{-6} M</td>
<td>1 ± 1</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>IL-3 + OAG</td>
<td>10^{-7} M</td>
<td>2 ± 1</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>IL-3 + OAG</td>
<td>10^{-8} M</td>
<td>2 ± 1</td>
<td>17 ± 2**</td>
</tr>
<tr>
<td>IL-3 + OAG</td>
<td>10^{-9} M</td>
<td>2 ± 1</td>
<td>17 ± 2**</td>
</tr>
<tr>
<td>IL-3 + OAG</td>
<td>10^{-10} M</td>
<td>2 ± 1</td>
<td>16 ± 2</td>
</tr>
</tbody>
</table>

Colonies were counted on day 8 and day 16. Data represent mean ± S.D. of quadruplicate dishes. Neither TPA or OAG alone gave rise to colony growth.

*P < .05, **P < .01 compared with IL-3 + vehicle control. +++ P < .01 compared with IL-3 + TPA (10^{-7} M).

### Table 2

**Differential counts of colonies**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Number of Colonies</th>
<th>GM</th>
<th>GEMM</th>
<th>Blast</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3 + vehicle</td>
<td>3 ± 1</td>
<td>7 ± 2</td>
<td>3 ± 1</td>
<td>13 ± 2</td>
<td></td>
</tr>
<tr>
<td>IL-3 + TPA</td>
<td>6 ± 1*</td>
<td>17 ± 1**</td>
<td>1 ± 1</td>
<td>24 ± 1**</td>
<td></td>
</tr>
<tr>
<td>IL-3 + OAG</td>
<td>4 ± 1</td>
<td>11 ± 4*</td>
<td>2 ± 1</td>
<td>17 ± 2***</td>
<td></td>
</tr>
</tbody>
</table>

Blast: blast cell colonies. Colonies were counted on day 16.

Data are expressed as the mean ± S.D. of quadruplicate cultures.

*P < .05, **P < .01 compared with IL-3 + vehicle. + P < .05, +++ P < .01 compared with IL-3 + TPA.

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Fig. 1. Time course of colony formation. Day-2 post 5-FU marrow cells were cultured with 2 U/ml of Ep and designated agents. Colonies were counted on every third day. Colony numbers show mean number for four dishes, each containing 5 x 10^4 cells.
Next we examined the involvement of PKC in the proliferation of primitive progenitors stimulated by IL-3 alone or in combination with TPA. When the cells were preincubated with a vehicle, a difference in colony numbers was observed between those supported by IL-3 and those supported by IL-3 + TPA. However, IL-3 and IL-3 + TPA gave rise to almost the same number of colonies (13 vs. 11) after preincubation with TPA (table 5). These results suggested that 48 hr of incubation with TPA may have down-regulated the PKC in the progenitor cells, resulting in the loss of the enhancing effect of TPA.

**Effects of preincubation with TPA alone and in combination with protein kinase inhibitors on IL-3-dependent colony formation.** To infer a signal transduction pathway of TPA, we preincubated Day-2 post 5-FU marrow cells for 12 hr with TPA alone or in combination with the inhibitors. After washing twice, the cells were cultured with IL-3. The specific PKC inhibitor calphostin C abrogated the enhancing effects of TPA at a concentration of 200 nM; this concentration was 4 times higher than the IC$_{50}$ value against PKC and much lower than the IC$_{50}$ value against PTK and A-kinase. Genistein and herbimycin A also abrogated the effects of TPA at 10 µg/ml and 200 ng/ml, respectively. This concentration of genistein was almost the same as that of the IC$_{50}$ against tyrosine kinase, and the concentration of herbimycin A was markedly lower than the concentration of IC$_{50}$ against tyrosine kinase (table 6). The incubation of Day-2 post 5-FU marrow cells for 12 hr with DMSO, calphostin C, genistein or herbimycin A had no effect on the viability of these cells. The viabilities of the Day-2 post 5-FU marrow cells incubated with DMSO (0.1%–0.001%), calphostin C (50–200 nM), genistein (1–100 µg/ml) and herbimycin A (50–200 ng/ml) were 97.9% to 98.4%, 98.9% to 99.0%, 96.9% to 99.2% and 98.1% to 98.7%, respectively. Although these results suggest that TPA augmented IL-3-dependent colony formation, probably through activation not only of PKC but also of certain tyrosine kinases, direct evidence of this activation of PKC and tyrosine kinase is needed for confirmation.

**TABLE 4**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Number of Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GM</td>
</tr>
<tr>
<td>IL-3 + vehicle</td>
<td>1</td>
</tr>
<tr>
<td>IL-3 + TPA</td>
<td>6</td>
</tr>
<tr>
<td>IL-3 + IL-6</td>
<td>4</td>
</tr>
</tbody>
</table>

Data represent mean ± S.D. of quadruplicate cultures. * P < .05, ** P < .01 compared with IL-3 + vehicle. ** P < .01 compared with IL-3 + TPA.

**TABLE 5**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Number of Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preincubated with Vehicle</td>
</tr>
<tr>
<td>IL-3 + vehicle</td>
<td>4</td>
</tr>
<tr>
<td>IL-3 + TPA</td>
<td>8</td>
</tr>
<tr>
<td>IL-3 + IL-6</td>
<td>6</td>
</tr>
</tbody>
</table>

1 × 10$^5$ marrow cells were preincubated with 2 × 10$^{-8}$ M TPA for 48 hr. After washing, cells were divided into four dishes. Colonies were counted on day 16.

Data represent numbers of total colonies in four dishes (colonies per 1 × 10$^5$ marrow cells).
programmed cell death of progenitor cells. It has been re-
cell CFC (figs. 1 and 2), it seems possible that TPA acts
the blast cell colonies or shorten the G0 period of the blast
other synergistic factors, did not hasten the appearance of
ened the G0 period of blast cell CFC. Because TPA, unlike
not influence the cell doubling time of blast cells but short-
IL-3-dependent colony formation, the synergistic factors did
appear to support this observation.
lar to that triggered by a single dose of TPA (Nishizuka,
ment of OAG is required to produce a cellular response sim-
OAG might be due to its rapid metabolism. Repeated supple-
1991), its mechanism is as yet unclear. The weaker action of
Nicola, 1983) or in combination with IL-6 (Heyworth
concentration has already been reported (Kanakura
of colonies. Although the suppressive effect of TPA at high
dose evaluated; at $10^{-8}$ M, there was a reduced number
of colonies. Although the suppressive effect of TPA at high
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mend the role of OAG is required to produce a cellular response sim-
ilar to that triggered by a single dose of TPA (Nishizuka,
1992). Our data from the addition of supplement OAG also
appear to support this observation.

TPA is well known to induce the monocytic differentiation
of myeloid leukemia cells, such as HL-60 (Rovera et al., 1979;
Huberman and Callaham, 1979) and KG-1 (Ferrero et al.,
1983). TPA, alone (Stuart and Hamilton, 1980; Burgess and
Nicola, 1983) or in combination with IL-6 (Heyworth et al.,
1993; Whetton et al., 1994), can also stimulate the prolife-ation of GM-CFC. But the extent of these stimulative effects of
TPA is not limited to GM lineage: as we have shown, TPA in
combination with IL-3 increased not only GM colonies but
also multilineage GEMM colonies (table 2).

In terms of the synergistic effects of synergistic factors on
IL-3-dependent colony formation, the synergistic factors did
not influence the cell doubling time of blast cells but short-
ened the G0 period of blast cell CFC. Because TPA, unlike
other synergistic factors, did not hasten the appearance of
the blast cell colonies or shorten the G0 period of the blast
cell CFC (figs. 1 and 2), it seems possible that TPA acts
through some other mechanism than the shortening of the
G0 period of progenitor cells, such as suppression of the
programmed cell death of progenitor cells. It has been re-
ported that not only IL-3 (Williams et al., 1990; Collins et al.,
1992) but also TPA can rescue bone marrow cells from apo-
poptosis through activation of PKC (Lotem et al., 1991).
Further studies are needed to confirm the relationship between
the rescue of progenitor cells by TPA from apoptosis and the
enhancing effect of TPA on IL-3-dependent colony formation
in primitive progenitor cells.

**Discussion**

In this study we showed that TPA directly augmented the
IL-3-dependent colony formation of primitive hematopoietic
progenitors through the activation of PKC and certain tyro-
sine kinases. TPA did not augment colony formation at all
the doses evaluated; at $10^{-8}$ M, there was a reduced number
of colonies. Although the suppressive effect of TPA at high
concentration has already been reported (Kanakura
1991), its mechanism is as yet unclear. The weaker action of
OAG might be due to its rapid metabolism. Repeated supple-
mend of OAG is required to produce a cellular response sim-
ilar to that triggered by a single dose of TPA (Nishizuka,
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In terms of the synergistic effects of synergistic factors on
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not influence the cell doubling time of blast cells but short-
ened the G0 period of blast cell CFC. Because TPA, unlike
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the blast cell colonies or shorten the G0 period of the blast
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Further studies are needed to confirm the relationship between
the rescue of progenitor cells by TPA from apoptosis and the
enhancing effect of TPA on IL-3-dependent colony formation
in primitive progenitor cells.

Next we examined whether TPA acted on primitive pro-
genitors directly or indirectly through accessory cells con-
tained in the cultures. To do this, we used blast cells obtained
from the blast cell colonies that had developed in the pres-
ence of IL-3. These pooled blast cells are not primitive pro-
genitor cells in themselves, however, they can give rise to
secondary colonies, including GEMM colonies (Nakahata and
Ogawa, 1982; Musashi et al., 1991a). The lower plating effi-
ciencies than those found in previous experiments might be
attributable to different culture conditions, such as the qual-
ity of fetal calf serum. TPA also enhanced the IL-3-dependent colony formation derived from lineage-negative Day-2 post
5-FU marrow cells. The multiple increase of colonies by TPA
from lineage-negative cells was higher than that from Day-2
post 5-FU marrow cells (6-fold vs. 1.8-fold).

Then we focused on the signal transduction of TPA in
IL-3-dependent colony formation derived from Day-2 post
5-FU marrow cells. First, we examined the effects on colony
formation of prolonged pretreatment of the progenitors with
TPA. Although we did not measure PKC activity in the bone
marrow cells before and after the prolonged incubation with
TPA, it has been reported that incubation with $2 \times 10^{-8}$ M
TPA for 48 hr resulted in a down-regulation of PKC (Nishi-
zuka, 1984; Goodnight et al., 1994). TPA did not enhance
IL-3-dependent colony growth further after 48 hr of incuba-
tion, which suggests the involvement of PKC in the enhanc-
ing effect of TPA on IL-3-dependent colony formation. Sec-
ond, we used protein kinase inhibitors against PKC and
against tyrosine kinase. In these studies, the specificity of
the inhibitors is critical. Calphostin C is a recently developed
PKC inhibitor. It works by binding to the regulatory domain
of PKC (Nishizuka, 1988; 1992) and does not share a common
homology with other protein kinases, so it gives specific in-
hibitory effects. Calphostin C has a 1000 times lower IC$_{50}$
value against PKC than against tyrosine kinase or A-kinase.
Because high concentrations of calphostin C have been re-
ported to be somewhat cytotoxic to human tumor cells (Bruns
et al., 1991), we examined the cytotoxic effects of calphostin C
on Day-2 post 5-FU marrow cells. Twelve hours of incubation
with calphostin C at a concentration of 50 to 200 nM did not
influence cell viability. The fact that calphostin C abrogated
the augmentation of IL-3-dependent colony formation by
TPA suggests that PKC might be involved in the enhancing
effects of TPA. We cannot, however, exclude the possibility
that calphostin C blocks the signaling not only of TPA but
also of IL-3, because calphostin C decreased colony numbers
to less than that achieved with IL-3 stimulation only.

Genistein and herbimycin A, specific tyrosine kinase inhibi-
tors, also inhibited the enhancing effects of TPA. One tyro-
sine kinase that may be activated by TPA and blocked by
these inhibitors is MAP kinase (Gilmore and Martin, 1983;
Vila and Weber, 1988), which is activated by both phosphor-
ylation of threonine and tyrosine residues. Because genistein
and herbimycin A, as well as calphostin C, completely abro-
gated IL-3-dependent colony formation, we could not ignore
the possibility that these tyrosine kinase inhibitors might
block the signaling of both TPA and IL-3, of which the intra-
cellular signal is transduced via tyrosine kinase JAK 2 (Sil-
vennoinen et al., 1993).

Further studies aimed at providing direct evidence of the
involvement of PKC in the enhancing effects of TPA on the

**Table 6**

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Number of Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>TPA</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>TPA + calphostin C</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>50 nM</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>100 nM</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>200 nM</td>
<td>1 ± 1**</td>
</tr>
<tr>
<td>TPA + genistein</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>0**</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>0**</td>
</tr>
<tr>
<td>TPA + herbimycin A</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>50 ng/ml</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>200 ng/ml</td>
<td>1 ± 1**</td>
</tr>
</tbody>
</table>

$5 \times 10^5$ Day 2-post 5-FU bone marrow cells were incubated with TPA either alone or in combination with inhibitors for 12 hr. After washing twice, cells were cultured with 100 U/ml of IL-3. In this experiment, 2-ME was not added to the cultures, because 2-ME has been reported to inactivate herbimycin A (Uehara et al., 1989). Colonies were counted on day 16 of culture. Data represent mean ± S.D. of quadruplicate cultures. **P < .01 compared with TPA.
IL-3-dependent colony formation of primitive hematopoietic progenitors are underway.

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Whetton, A.D., Heyworth, C.M. and Dexter, T.M.: Phorbol esters activate protein kinase C and glucose transport and can replace the requirement for


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