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ABSTRACT

There has been much recent interest in the role of a calcium-dependent, phospholipid-dependent protein kinase (PKC) in regulation of macrophage function and growth. In order to better define the role of PKC in these processes we have developed mutants of the RAW264 macrophage-like cell line that are resistant to the growth-inhibiting effects of 12-O-tetradecanoylphorbol-13-acetate (TPA), a potent stimulator of PKC activity. Concentrations of TPA of $10^{-7}$ M or greater resulted in more than 90% inhibition of growth of RAW264 cells and were used to select for TPA-response mutants. Ultraviolet mutagenesis of $10^6$ cells and growth selection under inhibitory TPA concentrations yielded 12 colonies. Two isolated lines (M11 and M12) were studied in detail. Neither cell line was deficient in, nor appeared to have an altered PKC, based on enzymatic activity in response to TPA or diacylglycerol. Both cell lines grew in the presence of $10^{-4}$ M TPA. After removal of TPA, mutant M11 continued to grow while M12 died. Growth of M12 cells was TPA concentration dependent. Flow cytometric analysis of the DNA content of M12 cells in the absence of TPA indicated that cell growth was arrested in $G_0$ or $G_1$. This was interpreted as indicating that TPA acted as a growth factor inducing cells to enter the cell cycle. M12 cells could also grow in L-cell-conditioned medium containing colony-stimulating factor-1 (CSF-1), the normal growth factor for cells of the macrophage lineage. After subcloning M12 cells, it was found that several subclones of M12 did not grow in response to TPA but did grow well in L-cell medium. These cells would grow in medium conditioned by the exposure of TPA-responsive subclones to TPA. Bone marrow culture cells also grew in the conditioned medium. The growth factors produced by the TPA-responsive subclones were not neutralized by anti-CSF-1 antiserum. These results suggest that TPA may not directly induce the growth of M12 cells or the formation of colonies of bone marrow cells, but instead may act through the induction of a non-CSF-1 growth factor. These mutants can be used as tools for future studies on the role of PKC in the regulation of macrophage growth.

INTRODUCTION

Macrophages can be induced to assume an activated state by a variety of agents. Phorbol esters, which are a group of plant diterpenes that can promote tumors in a number of cell types (1–5) have been shown to be potent macrophage activators. TPA,1 a potent tumor promoter in animals, induces a number of biological effects on cells in culture, which depend on the degree of differentiation of the cell under study. In macrophages and macrophage-like cell lines, TPA was shown to stimulate mRNA synthesis and cell proliferation (6), to enhance the synthesis of prostaglandins (7) and the secretion of plasminogen activator (8), and to cause alterations in cell membrane permeability. TPA also stimulates bone marrow cells in agar cultures to differentiate into macrophages (9).

Although the details of the mechanism of action of phorbol esters are not fully understood, they have been shown to function by selectively activating a Ca$^{2+}$-dependent, phosphatidyserine-dependent protein kinase, PKC, in the cell membrane (10, 11). Activation of PKC has been implicated in regulating cellular processes (12), including the release of hormones (13) and neurotransmitters (14), the alteration of ionic fluxes (15), production of superoxide (16), and the respiratory burst of macrophages (17).

To assess the involvement of PKC in macrophage function, we have sought to generate TPA-resistant mutants of a TPA-responsive murine macrophage-like cell line. It was demonstrated that TPA inhibited the growth of the parent line but not that of the mutants. The selected mutants had normal or elevated PKC activity. One of the variants was demonstrated to be dependent on TPA for growth, and in this paper we describe the selection, subcloning, and characterization of this cell line.

MATERIALS AND METHODS

Reagents. Phosphatidyserine, histone (type V-S), TPA, dimethyl sulfoxide, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). [γ$^{32}$P]ATP was obtained from ICN (Irvine, CA). Anti-CSF-1 antisera were a generous gift from Dr. E. R. Stanley (Albert Einstein College of Medicine, New York, NY). TPA was dissolved in dimethyl sulfoxide at a final concentration of $1 \times 10^{-4}$ M for PKC assays, or $10^{-3}$ M for cell propagation, and stored at $-70$°C for no more than 2 months.

Cell Propagation. The macrophage-like cell line, RAW264 (18), was grown in Petri dishes in DMEM (GIBCO, Grand Island, NY) supplemented with 1 mm sodium pyruvate, 20 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5 µg/ml gentamicyn, and 5% FBS (Hazelton Dutchland, Hazelton, PA). All tissue culture medium used tested negatively for contaminating endotoxin in the Limulus lysate assay. Mutant variants of RAW264 were grown in DMEM supplemented with 5% FBS and either $10^{-4}$ M TPA or 10% LCM. Bone marrow cells were obtained from C57/HeN mice and were grown in liquid culture DMEM containing 10% LCM and 15% FBS. Agarose (UltraTure, Bethesda Research Laboratories, Gaithersburg, MD) was added at a final concentration of 0.5% for colony assay (colony-forming units) of bone marrow cultures. All cells were incubated at 37°C under 5% CO$_2$ tension.

Measurement of Cell Growth. Kinetics of growth of RAW264 and mutants was measured by plating $2 \times 10^5$ cells in 2 ml of serum-containing medium in each well of 6-well tissue culture plates. Growth stimulants or inhibitors were added as indicated. Cells were removed from the wells by scraping and were counted using a hemocytometer. Bone marrow cell type was assessed from the liquid cultures by differential staining using Difquick (American Scientific Products).
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isolated and propagated, and samples were frozen for further characterization. The mutant line, M12, was subcloned by limiting dilutions followed by propagation in the presence of CSF-1.

Assay of Protein Kinase C Activity. Protein kinase C was extracted from cells lysed by vortexing on ice in a buffer solution containing 2 mM ethyleneglycol bis(β-aminoethyl ether)-N,N',N,N'-tetraacetic acid, 100 mM EDTA, 0.1% Triton X-100, and 2 mM phenylmethylsulfonyl fluoride. Protein kinase C activity was then assayed as previously described (19).

RESULTS

Selection of TPA-resistant Mutants of RAW264. Tumor promoters, such as TPA, have been shown to inhibit the growth of many types of tumor cells (20, 21). In order to select for a mutant of RAW264 that had an altered response to TPA, a concentration of TPA was determined that inhibited the growth of the cell line. Growth of RAW264 was retarded by TPA in a concentration-dependent manner (Fig. 1). Cell growth was inhibited by 50% after 3 days in culture at $10^{-8}$ M TPA. At $10^{-6}$ M TPA, the growth of the cells was retarded by more than 90%. TPA ($10^{-6}$ M) was chosen for selection of the mutants. A 10-fold increase in the concentration of TPA ($10^{-5}$ M) resulted in total cell lysis.

RAW264 cells were UV irradiated for 15 s and then grown in medium containing $10^{-6}$ M TPA. Mutants that survived TPA inhibition and grew rapidly were selected and further propagated. The characteristics of two mutants, M12 and M11, were studied in detail. Both mutant lines retained the functional characteristics of the parent RAW264 cell line including Fc-mediated phagocytosis and responsiveness to bacterial lipopolysaccharide (data not shown). Both mutant lines grew well in concentrations of TPA shown to inhibit the parent cell line (Fig. 2), even though their growth in TPA was slower than the growth of uninhibited RAW264 cells. TPA-treated M11 cells grew at 50% of the rate of untreated RAW264 cells, while the growth rate of M12 in TPA approached that of the uninhibited parent line. The two mutant lines responded differently to the removal of TPA from the medium. M11 grew equally well in the presence or absence of TPA. The growth of M12 was retarded by 90% when TPA was removed; the majority of cells lysed within 5 days and the remaining 10% showed marked morphological variation after 10 days in medium depleted of TPA. It appears, then, that M12 became dependent on TPA for its growth.

To assess the dependence of M12 on TPA for growth, we studied the effect of different concentrations of TPA in the growth medium. Fig. 3 shows that the TPA concentration used in selecting the mutant ($10^{-6}$ M) resulted in maximal stimulation of growth. A 10-fold lowering in concentration ($10^{-7}$ M) was only slightly less effective. Half-maximal growth was obtained with $10^{-5}$ M TPA. Further lowering of the concentration of TPA resulted in a proportional lowering in growth rate. M12 depended for its growth on TPA in a concentration-dependent manner, which was kinetically the reciprocal of that of the...
Growth of M12 in CSF-1. TPA has been shown to be capable of replacing CSF-1, the colony-stimulating factor in macrophages and macrophage-like cell lines, in inducing bone marrow cells to differentiate along the macrophage lineage (22–26). Since flow cytometric studies of cellular DNA indicated that M12 cells were dependent on TPA to progress through the cell cycle (data not shown), and CSF-1 has been shown to allow phenotype of M12, being responsive to both LCM and TPA, without added LCM or TPA. The majority of cells retained the phenotype of M12, which ceased growth in the absence of TPA, grew when either TPA or LCM, a source of CSF-1 (28), was present in the growth medium. High concentrations of LCM (CSF-1) were necessary to equal the effect of TPA. In addition, CSF-1 was rapidly depleted from the medium, as repeated additions were required to maintain growth, while TPA appeared to have a prolonged effect.

Subcloning of M12. M12 cells were subcloned by plating limiting dilutions of cells and growing these cells in medium with 10% LCM. Subclones were selected by determining their responses to LCM, TPA, or medium without growth factors. It was found that at least three phenotypes of subclones were present. One type had reverted and was able to grow in medium without added LCM or TPA. The majority of cells retained the phenotype of M12, being responsive to both LCM and TPA. Subclone M12.5 was chosen as a representative clone.

Another subtype grew well in the presence of LCM, but would not grow in medium without LCM or in response to TPA (Table 2). This phenotype, represented by clone M12.1, was presented by 40% of the subclones tested. In order to determine if TPA inhibited growth of clone M12.1, as with the parent RAW264 cells, M12.1 cells were treated with either CSF-1 or TPA. When both stimulating factors were present in the medium for several days before performing the PKC assay, Table 1 shows the activity of PKC using M12 and M12 cell lysates. There was a slight diminution of PKC levels in M12 cells 3 days after TPA treatment which increased to levels significantly higher than RAW264 parent cells after continued culture. PKC levels from M12 cells 3 days after TPA treatment which increased to levels significantly higher than RAW264 parent cells after continued culture. PKC activity was shown to be normal in response to diacylglycerol, a physiological stimulus of the enzyme, and the enzyme had a K_{m} for TPA similar to the reported value for PKC from other cells.

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Production of Growth Factors by M12 and M12.5. The original selection of M12 was made with TPA, but many of the subclones would not grow in response to TPA. We therefore decided to determine how these clones were originally derived. One possibility was that the TPA-responsive subclones of M12, such as M12.5, produce a growth factor in response to TPA that could then stimulate other cells in the population unable to produce this factor. To test this possibility, M12.5 cells were grown in TPA for 3 days, and removed from the conditioned media by centrifugation. The pH of the medium was adjusted to 7.3 and the conditioned medium was filter sterilized.

Table 1 | Protein kinase C activity of RAW264 and variants

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW264*</td>
<td>1</td>
</tr>
<tr>
<td>M11*</td>
<td>0.45</td>
</tr>
<tr>
<td>M11*</td>
<td>2.67</td>
</tr>
<tr>
<td>M12*</td>
<td>2.08</td>
</tr>
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</table>

* Cells (10^6) were lysed by vortexing on ice in a lysis buffer containing 2 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 100 mM EDTA, 0.1% Triton X-100, and 2 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged at 10,000 x g for 5 min. PKC activity was determined by incubating 20 μl of cell lysate with an assay buffer containing 25 mM Tris (pH 7.5), 2 μg/ml histone, 25 μg/ml phosphatidylserine, 5 μM ATP containing 1 μCi [γ-32P]P, 10 ng/ml TPA, and 10 mM CaCl2. The reaction mixture was incubated for 10 min at 30°C, and was terminated by adding 1 ml 10% ice-cold trichloroacetic acid. The mixture was washed twice with trichloroacetic acid and centrifuged. The protein was dissolved in 400 μl Protosol (New England Nuclear, Boston MA), and its radioactivity was assayed.

PKC activity was assayed after cells had been removed from TPA for 3 days.

PKC activity was assayed after cells had been removed from TPA for 8 days.

Table 2 | Growth of mutant 12.1 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells/ml x 10^6 ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium*</td>
<td>4 ± 0.816</td>
</tr>
<tr>
<td>TPA (10^-4 M)</td>
<td>5 ± 3.35</td>
</tr>
<tr>
<td>L-cell medium (10%)</td>
<td>115 ± 9.7</td>
</tr>
<tr>
<td>M12.5 cell medium*</td>
<td>31 ± 1.82</td>
</tr>
<tr>
<td>10%</td>
<td>42 ± 1.63</td>
</tr>
<tr>
<td>20%</td>
<td>51 ± 2.9</td>
</tr>
<tr>
<td>50%</td>
<td>51 ± 2.9</td>
</tr>
</tbody>
</table>

* Cells were plated at a density of 1 x 10^5 cells in 2 ml of DMEM with 5% FBS. Viable cells were counted 3 days later.

M12.5 cells were plated (5 x 10^5 cells) in DMEM with 5% serum and 10^-4 M TPA. After 3 days the medium was processed by centrifugation to remove cells, the pH was adjusted to 7.4, and the conditioned medium was sterilized by filtration.

Fig. 4. Growth of M12 in medium containing CSF-1. Cells were plated as described in Fig. 1 legend. The media were supplemented with 5% FBS (A), with TPA (B), or 10% LCM (C). LCM was added at days 0 and 2. Points, mean of 3 experiments.

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* Cells were plated at a density of 1 x 10^5 cells in 2 ml of DMEM with 5% FBS. Viable cells were counted 3 days later.

M12.5 cells were plated (5 x 10^5 cells) in DMEM with 5% serum and 10^-4 M TPA. After 3 days the medium was processed by centrifugation to remove cells, the pH was adjusted to 7.4, and the conditioned medium was sterilized by filtration.
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Table 3 Effect of anti-CSF-1 antisum

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells/ml x 10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium*</td>
<td>7.2 ± 1.2</td>
</tr>
<tr>
<td>L-cell medium</td>
<td>72 ± 8.7</td>
</tr>
<tr>
<td>L-cell medium + antibody (1:1000)</td>
<td>3 ± 1.1</td>
</tr>
<tr>
<td>M12.5 cell medium (50%)*</td>
<td>57 ± 6.8</td>
</tr>
<tr>
<td>M12.5 cell medium + antibody</td>
<td>55 ± 7.9</td>
</tr>
</tbody>
</table>

* M12.1 cells (1 x 10^6) were plated in DMEM containing 5% FBS. Cells were counted after 3 days.
* Anti-CSF antibody was added at a final concentration of 1:1000, a concentration that was determined to block the effect of LCM.
* Media conditioned by M12.5 were prepared as described in the text.

Table 4 Effect of conditioned medium on bone marrow cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells/ml x 10^4</th>
<th>Colony forming units/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium*</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>L-cell medium</td>
<td>4.5 ± 2.5</td>
<td>41.5 ± 8.5</td>
</tr>
<tr>
<td>TPA (10^(-6)M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TPA + antibody</td>
<td>0</td>
<td>ND*</td>
</tr>
<tr>
<td>M12.5 cell medium†</td>
<td>3.5 ± 1.2</td>
<td>30.3 ± 10.2</td>
</tr>
<tr>
<td>M12.5 cell medium + antibody</td>
<td>4.0 ± 1.7</td>
<td>32 ± 2</td>
</tr>
</tbody>
</table>

† freshly collected bone marrow cells (1 x 10^6) were plated in DMEM containing 15% FBS. Cells were counted after 14 days.
* Anti-CSF antibody was added at a final concentration of 1:1000, a concentration that was determined to block the effect of LCM.
* ND, not determined.
† M12.5 cell-conditioned medium was prepared as described in the text.

factor in M12.5-conditioned medium which is different from CSF-1. This growth factor was not produced by CSF-1-treated cells.

Growth of Bone Marrow Cells in M12-conditioned Medium. To determine if the growth factor produced by M12.5 cells could support the growth of normal cells, we grew bone marrow cells in M12.5-conditioned medium, with TPA or L-cell medium. Table 4 shows that LCM induced bone marrow cell colony formation. These were typical macrophage colonies by phase microscopy and by differential staining of the isolated cells. TPA was only able to cause the induction of a limited number of small macrophage colonies as assessed by analysis of the liquid cell culture but these were too few to count and did not appear in the semisoft agarose assay.

DISCUSSION

We have isolated TPA-resistant mutants of the TPA-sensitive macrophage-like cell line RAW264. All isolated mutants grew in the presence of TPA concentrations that were inhibitory to the parent cell line. We observed two patterns of response to the removal of TPA from the growth medium of the mutants, exemplified by mutants M12 and M11. M11 was not affected by the presence of TPA, while M12 was dependent on the presence of TPA. Both mutants had normal PKC activity in the absence of TPA.

These cells may have become resistant to TPA by a variety of mechanisms. One such mechanism is by reduction in TPA uptake. Since the initial step in TPA interaction is adherence to the cell membranes as a result of its highly lipophilic properties, it is possible that the mutants have acquired alterations in membrane properties that would result in reducing TPA incorporation into the membranes. Even though this mechanism may explain why the mutants became resistant to TPA, it cannot explain why M12 has become TPA dependent. The growth characteristics of M12 indicate that the sensitivity of these cells to TPA is not altered although the nature of their response is quite different. Other studies, such as ornithine decarboxylase induction, indicate that there is no alternation in the response of the mutant lines to TPA stimulation when compared to the parent RAW264 cell line.*

An alternative explanation to TPA resistance is altered expression of PKC, the only known cellular receptor for TPA (10). Two possible mechanisms may explain mutants resistant to TPA: reduction of the total number of active PKC molecules on the cell membrane, or alteration in molecular structure leading to reduced biological activity. Our studies have indicated that PKC activity in these cells appears normal. There is no alteration in the Kd of PKC for TPA and no loss of PKC if PKC activity is allowed to recover after down regulation by chronic TPA treatment. Initial “Western” immunoblotting studies using an antisum to PKC indicates that the molecular weight and the amount of PKC protein is not altered. This suggests that the molecular structure of PKC in the TPA-resistant variants and in RAW264 is not different.

TPA-dependent M12 cells will grow and divide in response to either TPA or LCM (a source of CSF-1), even though these agents have opposite effects on other responses in M12 cells, i.e., adherence to plastic plates and morphology of spreading. When M12 is plated in the absence of TPA or CSF-1 the cells fail to grow and given enough time the majority of cells will lyse. Thus, TPA appears to act by allowing M12 cells to passage G1 phase, a property previously attributed to CSF-1 in bone marrow cells (27). It is possible that the mechanism of action of either TPA or CSF-1 may be similar, perhaps indicating that PKC is involved in the action of CSF-1.

TPA has been reported to replace CSF-1 in a number of cells. We show here that this is also the case with the M12 cell line. Stuart et al. (9) discussed the possibility that TPA may substitute for CSF-1 as a growth factor. In those studies TPA effectively replaced CSF-1 as a growth factor for bone marrow colony cells. Anti-CSF-1 antibodies did not neutralize TPA stimulation of growth. It was concluded that the effect of TPA was not necessarily mediated by CSF-1, although other growth factors could not be ruled out. It is possible, then, that the function of the CSF-1 receptor is to induce phosphatidyl inositol turnover, thus activating PKC. TPA could then replace CSF-1 by directly activating the kinase.

A number of subcloned variants of M12, the mutant which was dependent on TPA for growth, grew in response to CSF-1 but not in response to TPA. Those subclones responded to TPA in other ways such as increased adherence and cellular spreading. Furthermore, the subcloned cells grew in the presence of both LCM and TPA, suggesting that TPA did not act as a growth inhibitor, as is the case with the parent RAW264. Thus, M12.1 cells represent a population of cells that have lost the ability to grow in response to TPA, but have retained an intact growth response to CSF-1. It, therefore, becomes difficult to implicate PKC in the CSF-1 response. These results suggest

4 Unpublished results.
5 S. M. Taffet and D. W. End, unpublished results.
that PKC activation cannot replace the interaction of CSF-1 with its receptor.

Our data indicate that cells that grew in response to TPA produced a growth factor. We do not know if production of this growth factor was the only mechanism by which cells grew in response to TPA. The growth factor was only produced in response to TPA and was capable of inducing growth in cells that did not grow in response to TPA alone. This is not in conflict with previous studies which showed that the induction of bone marrow colony formation by TPA was not inhibited by anti-CSF-1 antiserum (29). This observation, however, does not exclude the possibility that a non-CSF-1 growth factor was involved. In our case, it was also not possible to block the growth factor produced by M12 cells with neutralizing anti-CSF-1 antisera. Furthermore, the growth factor produced by M12 cells in response to TPA induced both macrophages and granulocytes. TPA has been shown to induce granulocyte-macrophage CSF production by macrophages (23). It is uncertain at this point whether this indirect mechanism of TPA action is a general mechanism in other cell types.

REFERENCES


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