Metabolism of Tritium-labeled 12-O-Tetradecanoylphorbol-13-acetate by Cells in Culture

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ABSTRACT

The metabolism of [20,3H]-12-O-tetradecanoylphorbol-13-acetate ([3H]TPA) was studied in human and hamster cell cultures. Within 2 to 3 days after its addition to growing or confluent cultures of hamster embryo fibroblasts, no unchanged [3H]TPA remained in the medium as determined by thin-layer chromatography of the chloroform phase obtained by extraction of the medium with chloroform:methanol:H2O. In contrast, little or no metabolism of [3H]TPA occurred under identical conditions in cultures of human fibroblasts. The major metabolite formed from [3H]TPA in hamster cell cultures was [3H]phorbol-13-acetate. With both hamster and human cells, virtually all cell-associated radioactivity was unchanged [3H]TPA.

INTRODUCTION

In vitro systems can provide valuable information on the mechanism of action of tumor promoters at the cellular and molecular levels. The phorbol diesters that act as promoters in the 2-stage model of mouse skin tumorigenesis also elicit a variety of biological responses when added to cell cultures. These include stimulation of cell growth (6,19) and DNA synthesis (17,23), loss of a large, cell membrane protein (8), production of plasminogen activator (21), induction of ornithine decarboxylase (14,23), and inhibition of cell differentiation (4,7,18,22). The induction of ornithine decarboxylase and stimulation of DNA synthesis also occur in mouse epidermis in vivo in response to treatment with tumor promoters described in these 2 papers, we can begin to answer this question.

MATERIALS AND METHODS

Cells. Primary hamster embryo cell cultures were prepared from 13-day-old Syrian hamster embryos (Lakeview Hamster Colony, Newfield, N. J.) as described (5). The human fibroblast line HC-4 was established in this laboratory from the normal conjunctiva of a 9-month-old infant with retinoblastoma. Cells were grown in plastic flasks (Falcon Plastics, Oxnard, Calif.) in Eagle's minimal essential medium (AutoPow; Flow Laboratories, Rockville, Md.) containing an additional mixture of vitamins as formulated for Eagle's basal medium and supplemented with 10% fetal bovine serum (Reheis Chemical Co., Chicago, Ill., or Flow Laboratories). The cells were routinely subcultured at a 1:4 ratio once or twice a week by trypsinization with a solution of 0.025% trypsin and 0.02% EDTA. HEP were used at the second or third passage, and HC-4 cells were used at passages 4 to 10 (population-doubling levels, 8 to 20).

Methods. The metabolism of [3H]TPA was determined as follows. Cells growing in T-75 flasks were exposed to [3H]TPA dissolved in 30 ml of medium to give a final concentration of 0.16 μM (1.28 μCi/ml). This concentration of [3H]TPA was not cytotoxic for either HEP or HC-4 cells. Aliquots of 0.5 ml medium were withdrawn after periods of time varying from 6 hr to 7 days and stored at -20°. The recovery of total radioactivity was monitored by direct high-pressure liquid chromatography recently confirmed that virtually no metabolism of [3H]TPA in mouse skin could be detected. Further studies by Kreibich et al. (9) did show considerable metabolism of 3 tritium-labeled phorbol diesters in short-term cultures of mouse L-cells and mouse skin explants but little or no metabolism by HeLa cells. The metabolism that did occur appeared to be loss of 1 of the 2 ester groups of TPA, yielding the monoester TP.

As part of a study of the biological responses to tumor promoters of various cells in culture, we have investigated the metabolism of phorbol diesters in hamster and human cell cultures by 2 independent methods. This paper describes the metabolism of [3H]TPA by these 2 cell types, and a second paper (15) describes a unique bioassay that does not require radioactive compounds for measuring the metabolism of TPA and other phorbol diesters. Although it has been assumed that the phorbol diesters do not require "metabolic activation" and that these compounds themselves are the actual biologically effective agents (3,9), this assumption has not been rigorously examined, especially in a tissue or cell culture system capable of metabolizing the compounds. By the use of a variety of cell culture systems and the techniques for measuring metabolism of promoters described in these 2 papers, we can begin to answer this question.

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2 To whom reprint requests should be addressed.
3 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; HEP, hamster embryo fibroblasts; TLC, thin-layer chromatography; PA, phorbol-13-acetate; TP, 12-O-tetradecanoylphorbol.
counting of aliquots of the culture media and was always 95% or greater over the 7-day experimental period.

For analysis, 0.2 ml aliquots of medium were extracted with chloroform:methanol:H2O (2:2:1.6) by the method of Baird and Diamond (1). The distribution of radioactivity between the aqueous methanol and chloroform phases was determined by liquid scintillation counting of 0.1-ml samples of each phase, and then the remainder of the chloroform phase was evaporated to a small volume and spotted on Eastman silica gel plastic TLC sheets with fluorescent indicator ("Chromagram"; Eastman Kodak Co., Rochester, N. Y.). Also included as standards on each plate were samples of nonradioactive TPA, PA, and TP. After development with methylene chloride:acetone (3:1), the standards were visualized either by short-wave UV or by vanillin:sulfuric acid reagent (11); the sheet was cut into 1-cm strips and eluted with 1.0 ml methanol; the radioactivity was determined by liquid scintillation counting in 7-ml polyethylene vials (Amersham/Searle Corp., Arlington Heights, Ill.) with 4 ml TT-21 scintillant (Yorktown Research, Inc., Hackensack, N. J.). Counting efficiencies were determined by the use of automatic external standard ratios.

Chemicals. [20-3H]TPA (8 Ci/mmol; greater than 95% radiochemically pure), TPA, PA, and TP were obtained from Dr. Peter Borchert, University of Minnesota, Minneapolis, Minn.

RESULTS

The procedure for organic extraction of cell culture medium used in these experiments resulted in 2 phases, an upper aqueous methanol phase and a lower chloroform phase. Chart 1 shows the distribution of radioactivity between the 2 phases after extraction of media samples from hamster and human cell cultures exposed to [3H]TPA for 7 days and from control flasks containing no cells but also incubated at 37°. With the HEF cultures, the percentage of radioactivity extractable in the chloroform phase decreased by approximately 50% after 3 days and then remained at this level. TLC (Chart 2) of the chloroform phases obtained at each time point from cultures of hamster cells showed that by 3 days most of the radioactivity extracted into chloroform was no longer [3H]TPA and that after 7 days no unchanged [3H]TPA was detectable. There was a corresponding increase in a radioactive peak cochromatographing with PA, the monoester derived by loss of the tetradecanoyl ester group at position 12 of TPA. No other peaks of radioactivity were detected.

The results obtained with the HC-4 human cells were very different. The distribution of radioactivity after extraction of the media from these cultures resulted in 2 phases, an upper aqueous methanol phase and a lower chloroform phase. Chart 1 shows the distribution of radioactivity between the 2 phases after extraction of media samples from culture media containing no cells (Chart 1). It was apparent from TLC analysis that virtually all the radioactivity in the chloroform phases of the media from both the human cell cultures (Chart 3) and the control flask (Chart 4) was unchanged [3H]TPA.

The previous experiments were done with growing cells, and even though the initial number of cells was similar the final number was much greater in the hamster than in the human cell cultures. To increase the number of cells initially exposed to [3H]TPA, confluent cultures of HC-4 and HEF were refed with fresh medium containing [3H]TPA, and a similar analysis was done. Under these conditions, the
Chart 3. Radiochromatograms of the chloroform extracts of media from HC-4 cells exposed to [3H]TPA for the indicated lengths of time. The samples were those described in Chart 1. Solvent system was methylene chloride:acetone (3:1).

Chart 4. Radiochromatograms of the chloroform extracts of control media containing [3H]TPA but no cells. The samples were those described in Chart 1. Solvent system was methylene chloride:acetone (3:1).

Confluent human cell cultures still showed only slight metabolism of [3H]TPA. The chloroform-extractable radioactivity in the medium slowly declined to 88% after 7 days of incubation compared to 93% in control medium without cells. TLC analysis (not shown) showed that even after 7 days more than 85% of the chloroform-extractable radioactivity was unchanged [3H]TPA compared to 92% in control medium; most of the remaining radioactivity in both cases cochromatographed with PA. Thus there was no appreciable difference in [3H]TPA metabolism in cultures of confluent as compared to cultures of growing hamster or human cells.

With both cell types, only 2 peaks of radioactivity were found in the chloroform phase, 1 corresponding to unchanged [3H]TPA and 1 to [3H]PA. This radioactive metabolite peak cochromatographs with authentic marker PA in 2 other solvent systems: anhydrous ether (4 successive developments); and ethyl acetate:chloroform (2:1).

The identity of the radioactivity in the aqueous phase of medium extracts from the hamster cells was determined in 2 ways: (a) the aqueous phase was reextracted with fresh chloroform, and the resulting chloroform phase was cochromatographed in methylene chloride:acetone (3:1); and (b) the aqueous phase itself was chromatographed in several solvent systems. As shown in Table 1, the radioactive material in the aqueous phase partitioned each time the aqueous phase was reextracted with fresh chloroform; the material obtained after reextraction cochromatographed with PA (Chart 5, top). Direct analysis of the aqueous phase by TLC showed 2 areas of radioactivity, a major peak cochromatographing with PA and a minor peak at the origin (Chart 5, bottom). Preliminary evidence from several TLC systems suggests that most of the material at the origin is [3H]phorbol, the unesterified parent compound of TPA.

Because it is possible that the [3H]TPA metabolites within the cells differed from those in the culture medium, the nature of the radioactivity remaining associated with intact cells was determined (Table 2). With both cell types, 99% of the cell-associated radioactivity was chloroform extractable, and virtually all was unchanged [3H]TPA, although a

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>dpm</th>
<th>%</th>
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<tbody>
<tr>
<td>1st extraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroform phase</td>
<td>3.06 × 10^6</td>
<td>54.8</td>
</tr>
<tr>
<td>Aqueous methanol phase</td>
<td>2.51 × 10^6</td>
<td>45.2</td>
</tr>
<tr>
<td>2nd extraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroform phase</td>
<td>0.99 × 10^6</td>
<td>44.6</td>
</tr>
<tr>
<td>Aqueous methanol phase</td>
<td>1.25 × 10^6</td>
<td>55.4</td>
</tr>
<tr>
<td>3rd extraction</td>
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<td></td>
</tr>
<tr>
<td>Chloroform phase</td>
<td>4.0 × 10^6</td>
<td>38.8</td>
</tr>
<tr>
<td>Aqueous methanol phase</td>
<td>6.3 × 10^4</td>
<td>61.2</td>
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minors of radioactive activity did not migrate with the solvent front of the chromatogram of the chloroform extracts from human cells (Chart 6). It appears that, once metabolism of [3H]TPA occurs, most of the metabolites produced are found in the medium.

DISCUSSION

There is increasing evidence that the tumor-promoting phorbol diesters can influence several important properties of cells in culture including transformation (12, 13), differentiation (4, 7, 18, 22), and the expression of mutations (20). However, it is not known whether the phorbol diesters themselves or their metabolites are responsible for these effects. This study describes the striking difference in the ability of human and hamster fibroblasts in culture to metabolize [3H]TPA. Hamster cells metabolized all the [3H]TPA in the medium within 2 to 3 days, whereas the human fibroblasts metabolized [3H]TPA only slightly, if at all, even over a 7-day period. Interestingly, even in the hamster cultures only 1 metabolite was found, [3H]PA, 1 of the 2 possible monoester derivatives of [3H]TPA. Once formed, [3H]PA appears to be stable and does not undergo further metabolism.

Kreibich et al. (9) reported significant metabolism of [3H]TPA in mouse cell cultures but identified the major metabolite as [3H]TP, which we did not find. In each of the 3 TLC solvent systems used in this study, PA had a greater mobility (higher R value) than did TP; this is the opposite of the order of mobility for these compounds reported by Kreibich et al. (9) with 1 of the same TLC systems. Although TP might be expected to migrate further in the relatively nonpolar solvents used because of its long-chain aliphatic ester group at the secondary C-12, this factor appears not to be as important a determinant of mobility as esterification, even with an acetate group, at the tertiary C-13 (P. Borchert, personal communication). In any case, we have

Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Radioactivity (dpm)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEF Aqueous methanol</td>
<td>8 x 10^4</td>
<td>1.0</td>
</tr>
<tr>
<td>HEF Chloroform phase</td>
<td>7.6 x 10^4</td>
<td>99.0</td>
</tr>
<tr>
<td>HC-4 Aqueous methanol</td>
<td>1.7 x 10^4</td>
<td>0.7</td>
</tr>
<tr>
<td>HC-4 Chloroform phase</td>
<td>25.8 x 10^4</td>
<td>99.3</td>
</tr>
</tbody>
</table>
found with 2 different samples of PA the same relative mobility (i.e., PA greater than TP) on both plastic and glass silica gel TLC plates. Further evidence that the metabolite produced in HEF is PA is that the metabolite partitions between the organic and aqueous phases after extraction.

The lack of metabolism of [3H]TPA by the human cell line HC-4 is intriguing. Kreibich et al. (9) reported previously that HeLa cells, a human carcinoma cell line, did not metabolize [3H]TPA during a 12-hr incubation. Why a given cell is able to metabolize phorbol diesters and whether this capability influences its biological responsiveness to such agents are not known.

Although the methods described here for measuring [3H]TPA metabolism are relatively simple, quick, and inexpensive, their usefulness is somewhat restricted. The methods cannot be applied to other phorbol diesters because they are not generally available with a radioactive label. Also, the technique gives no information about the biological activity of the metabolites formed. To overcome these problems, a bioassay for analyzing the metabolism of the phorbol diesters has been developed (15). The combined use of these 2 procedures will make it possible to determine for a given promoter if metabolism occurs in a particular cell culture system, what the metabolites are, and which chemical species is responsible for the specific cellular changes observed.

ACKNOWLEDGMENTS

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REFERENCES

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