Tumor Promoter Actions on Rat Embryonic Development in Culture

Brian E. Huber2 and Nigel A. Brown3

Department of Pharmacology, The George Washington University Medical Center, Washington, D. C. 20037

ABSTRACT

The many embryonic and developmental features associated with tumor promotion have prompted us to investigate the effects of a series of phorbol esters and related diterpene tumor promoters on mammalian embryogenesis. A culture system which supports the normal development of 10.4 day organogenesis-phase rat conceptuses was utilized. In this system, 12-O-tetradecanoylphorbol-13-acetate (TPA), a potent Stage I and II promoter, disrupted the morphology and function of the embryonic visceral yolk sac (dose required to affect 50% of conceptuses, 18 nm). The effect was characterized by an abnormal, progressive separation of the two cellular layers of the yolk sac, but cellular differentiation appeared to be uninterrupted. Parallel log dose-response lines for this effect were produced by phorbol-12,13-dibenzoate (dose required to affect 50% of conceptuses, 200 nm) and phorbol-12,13-diacetate (dose required to affect 50% of conceptuses, 300 nm) which are consistent with structure-activity relationships for other promotional actions of these compounds. In addition, the weak Stage I promoter, 4-O-methyl-12-O-tetradecanoylphorbol-13-acetate, produced identical effects but was 1400 times less potent than was TPA, while mezerein, a potent Stage II promoter, was as potent as was TPA. These observations support the hypothesis that embryonic cells may be differentially sensitive to early- and late-stage promoters. Ethylphenylpropionate, a nonpromoting hyperplastic agent in mouse skin, had no effect on yolk sac morphology or function at its maximum solubility (1.85 nm). Yolk sac disruption by TPA was potentiated by heat inactivation (56°, 30 min) or 0.45-μm filtration of the culture medium. A more advanced stage of yolk sac development was less sensitive to TPA disruption since 11.4 day conceptuses, which were cultured for 30 hr, developed identical lesions, but TPA was at least 5-fold less potent.

Thus, the tumor promoter-induced lesions of the rat yolk sac have some features consistent with late-stage tumor promotion and do not appear to be associated with general toxicity, hyperplasia, or alterations in cellular differentiation. We postulate that rat conceptuses maintained in vitro may provide an important model system for the study of the proposed mechanisms involved in chemical tumor promotion.

INTRODUCTION

Tumor promoters are noncarcinogenic compounds that enhance tumor formation in tissues previously treated with a subthreshold dose of a carcinogen. Promoters are of great importance because they are thought to be major determinants in certain human cancers (43, 44). Mouse skin has been the most extensively studied model system for investigating this 2-step scheme of chemical carcinogenesis, and the plant-derived diterpene phorbol esters are the most widely studied promoters in this system (11). Of all the phorbol esters, TPA4 is the most potent promoter of epidermal carcinogenesis (16, 41) and is commonly used as the model compound of its class. TPA exhibits a multitude of pleiotropic effects on mouse epidermal tissue (30, 32, 34) as well as cells in culture (3, 12, 42), but it is not known which of these responses is/are essential for the promotional process.

Several facts suggest the involvement of embryonic-like features in tumor promotion. When TPA is applied to mouse epidermis, there is the loss of chalone responsiveness (23), a decrease in histidase activity (9), an increase in both protease (40) and ornithine decarboxylase (29) activity, and the appearance of 2 embryonic proteins (2). All of these changes are characteristic features of embryonic skin (36). In addition, skin promotion has been separated into at least 2 distinct stages, originally described as conversion and propagation (4) but now termed Stage I and Stage II (35). Promoters may be active in one or both stages (35). The induction of dark basal keratinocytes, which are thought to be primitive stem cells normally abundant in embryonic skin, has been proposed as one important event in Stage I (37). These primitive stem cells may be especially sensitive to subsequent (Stage II) actions of tumor promoters. From these data, it has been suggested that, in general, cells of embryonic origin, such as the C3H/10T½ cell line, may require only Stage II promoters for complete promotional effects (35). Finally, it has been suggested that TPA may mimic certain properties of the polypeptide-transforming growth factors and, further, that these growth factors are involved in embryonic development (39).

In spite of the many embryonic features involved in promotion mentioned above, little is known about the interaction of tumor promoters with embryonic development. TPA has been reported to disrupt sea urchin embryonic development by blocking the differentiation of gut and skeletal cells (6). Effects on 4-cell stage frog embryos exposed to TPA included a dissociation of surface epithelial cells, acceleration of morphogenetic movements, and an inhibition of muscle segment histogenesis (13). Finally, TPA has been shown to delay cell growth and cause premature cell differentiation in preimplantation mouse embryos in culture (32). However, the relationship of these effects to those involved in tumor promotion are not clear since structure activity studies were not performed and nonmammalian systems were predominately used.

1 Supported by NIH Grants CA 32306-01 and RR 5359-20 Project 3-82. Presented in part at the Federation of American Societies for Experimental Biology Meeting, 1982, and the Joint Meeting of the American Society for Pharmacology and Experimental Therapeutics and the Society of Toxicology, 1982 (20, 21). This paper is the first in a series.

2 Predoctoral student in the Department of Pharmacology, George Washington University, Washington, D. C. To whom requests for reprints should be addressed.

3 Present address: Medical Research Council Laboratories, Carshalton, Surrey, SM5 4EF. United Kingdom.

Received February 22, 1983; accepted July 13, 1983.

4 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PDB, phorbol-12,13-dibenzoate; PDA, phorbol-12,13-diacetate; 4-O-MeTPA, 4-O-methyl-12-O-tetradecanoylphorbol-13-acetate; MZ, mezerein; EPP, ethylphenylpropionate; DMSO, dimethyl sulfoxide; p.c., post coitum; VYS, visceral yolk sac; ED50, dose required to affect 50% of conceptuses.
inantly used in these studies.

The overall purpose of the current program is to investigate the interactions of tumor promoters with mammalian embryogenesis and examine the suggestion that embryonic cells may be differentially sensitive to Stage I and Stage II promoters. In the present study, whole rat embryos in culture are utilized as the model system. This system allows the study of mammalian embryos undergoing normal growth and differentiation but without the complicating factors of the maternal environment.

MATERIALS AND METHODS

Chemicals. TPA, PDB, PDA, and 4-O-MeTPA were from Consolidated Midland Corp. (Brewster, N. Y.); TPA was also purchased from Chemical Carcinogenesis Research, Inc. (Eden Prairie, Minn.) as was MZ; EPP was from Aldrich Chemical Co. (Milwaukee, Wis.); JB-4 plastic for tissue embedding was from Polyscience, Inc. (Warrington, Pa.); and Coomasie Brilliant Blue was from Eastman Kodak (Rochester, N. Y.).

Animals. Timed-pregnant Sprague-Dawley rats were purchased from Zivic-Miller Laboratories (Allentown, Pa.) and were housed in climate-controlled conditions (20°, 7 a.m. to 7 p.m. light cycle) with free access to water and Purina rat chow.

Embryo Culture. Unless otherwise stated, dams were killed by cervical dislocation at 10.4 days p.c. (0 is the midpoint of the dark cycle during which mating occurred), and the uteri were removed. Conceptuses, composed of an early-headfold embryo, amnion, and chorion surrounded by the VYS, were explanted from uteri and aseptically dissected free of decidua, Reichert’s membrane, and the parietal yolk sac in sterile Hank’s balanced salt solution. Two conceptuses were cultured in 1 ml of 80% homologous rat serum in Waymouth’s medium contained in stoppered 30-ml serum bottles. Serum was obtained from ether-anesthetized male homologous rat serum in Waymouth’s medium contained in stoppered 30-ml serum bottles. Serum was obtained from ether-anesthetized male rats by exsanguination from the descending aorta. Serum was immediately centrifuged (28) and, unless stated, heat inactivated for 30 min at 56°(28) and filtered through 0.45-μm Millipore membrane filters. During the 30-hr culture period, bottles were rotated in a roller apparatus, and the temperature was maintained at 37°. The O₂ concentration of the gas phase was increased from an initial 20% to 40% at 8 hr (5% CO₂ at all times, the balance being N₂) by regassing the culture bottles.

Treatment. All compounds, dissolved in freshly distilled DMSO (TPA, PDA, PDB, 4-O-MeTPA, and EPP) or 0.1% ethyl acetate-99.9% acetone (MZ), were added directly to the culture medium 2 hr after the start of culture, in volumes not exceeding 2.5 μl. DMSO or ethyl acetate-acetone had no effect on viability or normal development of conceptuses at 20 or 15 μl per 4 ml culture medium, respectively. Conceptus viability and normal development were measured by heartbeat, VYS circulation, and developmental parameters of somite number, head and crown-rump length, morphological development score (7), and protein content (5). DMSO (2.5 μl/4 ml culture medium)- and/or ethyl acetate-acetone (2.5 μl/4 ml culture medium)-treated conceptuses were utilized as controls in all experiments.

Histology. Tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), dehydrated through 60% ethanol, and then infiltrated and embedded in JB-4 plastic. Specimens were sectioned at 1.5 μm using glass knives and were stained with hematoxylin and eosin.

RESULTS

Development of Control Rat Conceptuses In Vitro. In the culture system described, control conceptuses develop extensively, with pronounced organogenesis and cellular differentiation occurring within the 30-hr culture period. Over 400 control conceptuses were cultured in this study with 100% viability, and all developmental parameters which were defined in “Materials and Methods” were normal.

The VYS is the outermost membrane of the conceptus, as prepared for culture. At the start of culture (10.4 days p.c.), the VYS is at the rudimentary blood island stage, with few erythroblastic cells, no blood vessels, a total protein content of approximately 12 μg, and a diameter of approximately 2.1 mm. After 8 hr in culture, the VYS has increased to a diameter of approximately 2.5 mm (Fig. 1A). By the end of the 30-hr culture period, the VYS has developed a complete plexus of blood vessels (Fig. 1B) with functioning circulation of erythroblasts and has increased in protein content and diameter to approximately 120 μg and 5.0 mm, respectively. Throughout this entire 30-hr culture period, the VYS is composed of 2 tightly adhered cell layers, mesodermal and endodermal, between which the endothelial-lined vessels develop (Fig. 2A, and B).

The embryo itself, which develops inside the VYS, undergoes marked growth and differentiation throughout this 30-hr culture period (see Ref. 7 for more detail). Embryonic protein and crown-rump length, parameters of embryonic growth, increase from approximately 30 μg and 1.7 mm at 10.4 days p.c. to approximately 230 μg and 4.1 mm, respectively, by the end of the culture period. Morphological score (7) and somite number, parameters of embryonic differentiation and development, increase from approximately 10.4 and 8.5 at 10.4 days p.c. to approximately 41.2 and 28.0, respectively, by the end of the culture period. The morphological score is based upon objective classification of developmental stages of 17 embryonic organ primordia.

Tumor Promoter Effects on Rat Conceptuses. In this culture system, TPA, a potent Stage I and Stage II promoter (35), at 50 nw (all experiments use TPA from Consolidated Midland Corp., Batch 26, unless otherwise stated) caused blebs and swellings in the VYS, first visible by approximately 6 hr after conceptus treatment (Fig. 1A). By 28 hr, TPA-treated VYS appeared smooth and avascular with no circulatory system (Fig. 1B). Histological examination of these TPA-treated VYS revealed the progressive separation of the endoderm and mesoderm layers and the lack of formation of organized endothelial-lined vessels (Fig. 2, C to F). Endothelial cells are seen, however, within the 2 separated layers, with randomly dispersed primitive erythroblasts throughout the interlayer space. Endothelial cells were identified based on distinctive cell shape and staining properties. There was no indication of any areas of focal necrosis in either the endodermal or mesodermal layers of treated VYS. There does not appear to be any difference in the mitotic activity of the endodermal layer of TPA-treated and control VYS. The mitotic indices (expressed as the number of mitotic figures per 100 cells with 1000 consecutive cells counted) in the endodermal layer of control and TPA-treated VYS were 1.7 and 1.5, respectively, at the end of culture. Due to cellular morphology, mitotic figures cannot be easily observed in the mesodermal layer; thus, mitotic indices cannot be accurately estimated from standard slides in this VYS cell layer.

From these observations, a TPA-affected conceptus was defined as one with a VYS with no observable blood vessels and no circulating blood elements. At 50 nw TPA, 97% of treated conceptuses were affected. Interestingly, the few remaining VYS were morphologically and functionally normal. In other dose groups, VYS with a partial separation of the cellular layers were occasionally observed at the end of the culture period; however, the overall response to TPA seems to be close to an all-or-none
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The log dose-response curve for the morphological disruption of the VYS by TPA was steep, with 50% of the conceptuses (ED\textsubscript{50}) being affected at 18 nm (Chart 1). Reports have indicated differences in potency and biological characteristics of TPA (ED\textsubscript{50}) being affected at 18 nm (Chart 1). In contrast, the potent Stage II promoter, MZ, also produced identical lesions but was as potent as TPA in disrupting VYS development, with an ED\textsubscript{50} of 20 nm (Chart 1). EPP had no effect on the VYS at concentrations up to 1.85 \textit{nm}, which was the solubility limit in the culture medium.

Identification of other tumor promoters on this system, we have cultured conceptuses in the presence of PDB, a weak complete promoter (1, 17); PDA, a very weak complete promoter (1, 33) or non-promoter (35); 4-O-MeTPA, a weak Stage I promoter (33); MZ, a potent Stage II promoter (35); and EPP, a non-promoting epidermal hyperplastic agent (31, 35).

Illustrative VYS lesions and steep log dose-response curves were produced by PDB and PDA with ED\textsubscript{50}'s at 200 and 300 nm, respectively (Chart 1). The weak Stage I promoter, 4-O-MeTPA, produced the same effects but was approximately 1400 times less potent than was TPA, with an ED\textsubscript{50} at 25,000 nm (Chart 1). In contrast, the potent Stage II promoter, MZ, also produced identical lesions but was as potent as TPA in disrupting VYS development, with an ED\textsubscript{50} of 20 nm (Chart 1). EPP had no effect on the VYS at concentrations up to 1.85 nm, which was the solubility limit in the culture medium.

In those conceptuses which were treated with the above complete or stage-specific tumor promoters but which did not develop VYS lesions, development of the embryo itself appeared to be normal, regardless of the dose of promoter. Included in this assessment were the embryonic parameters of somite number, crown-rump length, head length, embryonic protein content, and morphological development score (representative data shown in Table 1). However, embryos were severely malformed and growth retarded if they developed within VYS that were classified as being affected (data not included; see "Discussion" for explanation; affected VYS defined in Chart 1 and Table 1 to 3).

Developmental Stage versus Potency. Additional conceptuses were explanted at 11.4 days p.c. and cultured for 30 hr, these conceptuses being 24 hr more advanced in development and having complete VYS blood vessel development and circulation at the start of culture. Treatment with TPA caused similar separation of the VYS cellular layers; at the end of the culture period, there were no macroscopically observable blood vessels. However, TPA was approximately 5-fold less potent in these developmentally advanced conceptuses (ED\textsubscript{50} 100 nm; Table 2). It is not clear whether the apparent disappearance of blood vessels is due to a physical disruption caused by the separation of VYS cellular layers or to some other nonspecific action of TPA.

Effects of Media Preparation on the Actions of TPA. Recent reports have indicated that certain serum components (19) or methods of media preparation (27) can alter the effects of TPA. Our serum preparation routinely included heat inactivation (56\textdegree C for 30 min) and 0.45-\mu m filtration prior to culture (see "Materials and Methods"). Both these procedures affected the actions of TPA on the VYS. A dose of 50 nm TPA was approximately 100% effective if the media was both heat inactivated and filtered but only 25% effective if either procedure was excluded (Table 3). In addition, if both procedures were excluded, 50 nm TPA had no effect on the VYS of cultured rat conceptuses (Table 3).

DISCUSSION

This study has demonstrated that TPA alters the functional development and organization of the developing rat conceptus by causing the abnormal progressive separation of the 2 VYS cell layers. The effect is dose dependent at nm concentrations of TPA.

One major problem involved in the study of the mechanism of promotion is distinguishing between those effects of promoters...
Table 1
Effects of phorbol diester derivatives on development of cultured rat embryos

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (μg/ml)</th>
<th>Unaffected conceptuses/total conceptuses cultured</th>
<th>Somites</th>
<th>Crown-rump length (mm)</th>
<th>Head length (mm)</th>
<th>Morphological score</th>
<th>Embryonic protein (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO (control)</td>
<td>2.5</td>
<td>104/104</td>
<td>28.0 ± 0.3 (28)</td>
<td>4.1 ± 0.1 (62)</td>
<td>2.0 ± 0.1 (44)</td>
<td>41.2 ± 0.2 (62)</td>
<td>229.6 ± 7.2 (41)</td>
</tr>
<tr>
<td>TPA</td>
<td>10</td>
<td>34/39</td>
<td>28.0 ± 0.3 (24)</td>
<td>3.9 ± 0.1 (24)</td>
<td>2.0 ± 0.1 (24)</td>
<td>40.0 ± 0.1 (24)</td>
<td>253.0 ± 9.5 (19)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1/6</td>
<td>29.0 (1)</td>
<td>4.2 (1)</td>
<td>2.2 (1)</td>
<td>40.0 (1)</td>
<td>223 (1)</td>
</tr>
<tr>
<td>PDB</td>
<td>50</td>
<td>3/88</td>
<td>29.0 ± 1.6 (3)</td>
<td>4.0 ± 0.1 (3)</td>
<td>1.9 ± 0.3 (3)</td>
<td>37.5 ± 1.8 (3)</td>
<td>ND</td>
</tr>
<tr>
<td>125</td>
<td>6/6</td>
<td>27.9 ± 0.9 (6)</td>
<td>4.3 ± 0.4 (6)</td>
<td>2.1 ± 0.1 (6)</td>
<td>39.2 ± 1.1 (6)</td>
<td>226.1 ± 17.7 (5)</td>
<td></td>
</tr>
<tr>
<td>175</td>
<td>2/6</td>
<td>27.5 (2)</td>
<td>4.2 (2)</td>
<td>2.0 (2)</td>
<td>36.5 (2)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>PDA</td>
<td>100</td>
<td>14/14</td>
<td>28.6 ± 0.3 (14)</td>
<td>4.1 ± 0.2 (14)</td>
<td>2.1 ± 0.1 (14)</td>
<td>40.4 ± 0.2 (14)</td>
<td>215.6 ± 11.1 (8)</td>
</tr>
<tr>
<td>256</td>
<td>11/16</td>
<td>28.7 ± 0.3 (11)</td>
<td>3.7 (2)</td>
<td>1.9 (2)</td>
<td>41.0 (2)</td>
<td>185.6 (2)</td>
<td></td>
</tr>
<tr>
<td>4-O-MeTPA</td>
<td>3,750</td>
<td>2/2</td>
<td>28.0 (2)</td>
<td>4.2 ± 0.2 (13)</td>
<td>2.2 ± 0.1 (11)</td>
<td>413.0 ± 0.2 (11)</td>
<td>238.0 ± 13.2 (13)</td>
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<td>20,000</td>
<td>27/28</td>
<td>28.7 ± 0.3 (13)</td>
<td>4.3 ± 0.1 (3)</td>
<td>2.2 ± 0.0 (3)</td>
<td>40.3 ± 0.3 (3)</td>
<td>220.3 ± 6.9 (3)</td>
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<tr>
<td>24,000</td>
<td>3/6</td>
<td>28.7 ± 0.4 (3)</td>
<td></td>
<td></td>
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</table>

*See Brown and Fabro (7).  
†Protein was determined by the method of Bradford (5).  
‡Mean ± S.E.; not determined for groups of less than 3. No values were significantly different from DMSO control values (p > 0.05, Newman-Keuls test).  
§Numbers in parentheses, number in group.  
ND, not determined.

Table 2
Effects of TPA on cultured rat conceptuses at different stages of gestation

<table>
<thead>
<tr>
<th>Dose of TPA (nm)</th>
<th>Embryonic age of explants (days p.c.)</th>
<th>Affected conceptuses/total conceptuses cultured</th>
<th>% affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>10.4</td>
<td>85/88</td>
<td>97</td>
</tr>
<tr>
<td>11.4</td>
<td>0/20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>10.4</td>
<td>49/49</td>
<td>100</td>
</tr>
<tr>
<td>8/16</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Control conceptuses at 10.4 and 11.4 days p.c., treated with 2.5 µM DMSO, were cultured and incubated identically to TPA-treated conceptuses; 118 control conceptuses were cultured with 100% viability (data not included, viability defined in "Materials and Methods").  
°0 is the midpoint of the dark cycle during which mating occurred.

Table 3
Differential actions of 50 nm TPA on rat conceptuses in various culture media

<table>
<thead>
<tr>
<th>Serum preparation</th>
<th>Heat inactivation*</th>
<th>Affected conceptuses/total conceptuses cultured</th>
<th>% affected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>++</td>
<td>93/96</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>4/16</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>3/14</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>0/8</td>
</tr>
</tbody>
</table>

*Heat inactivation and filtration described in "Materials and Methods." Heat inactivation or filtration had no effect on viability of DMSO (2.5 µM)-treated control conceptuses (data not included, viability defined in "Materials and Methods").

which are essential and specific for promotion and the many effects of these compounds that are nonessential and/or non-specific. The structure-activity relationships to disrupt VYS function within the series of phorbol diesters correspond to their ability to promote tumor formation in vivo (1). TPA is most potent in vivo (1, 16), PDB is less potent (1, 33), while PDA has been reported to be a very weak promoter (1, 33) or nonpromoter (35) in vivo. Interestingly, Slaga et al. (35) reported that 4-O-MeTPA, a nonpromoter in vivo (18, 35), was ineffective as a Stage II promoter at all doses tested but had weak but significant Stage I promoter properties at doses only 12 times that needed for TPA to exhibit Stage I effects. In our culture system, 4-O-MeTPA was approximately 140 times less potent than TPA in disrupting VYS morphology which is more consistent with a relationship of this VYS effect to Stage II promotional events than to Stage I events. Most striking is the fact that the nonphorbol compound, MZ, a potent Stage II promoter (35), was equipotent to TPA, again suggesting a relationship between VYS disruption and Stage II promotion. This supports the hypothesis that embryonic cells may be differentially susceptible to stage-specific promoters and may require only Stage II promoters for complete promotional effects (35). However, the confirmation of this hypothesis will require the study of a wider range of stage-specific promoters.

The morphological changes produced by TPA on the VYS do not appear to be related to either hyperplasia or cytotoxicity. Histological evaluation, mitotic indices, EPP results, and overall VYS growth do not indicate a major proliferative response or significant cellular necrosis. 4-O-MeTPA, shown to be as cytotoxic as TPA in certain cell culture systems (22), was approximately 1400 times less potent at VYS disruption, also suggesting a noncytotoxic effect. In addition, it appears that more advanced stages of VYS development are less sensitive to TPA disruption (see Table 2). This effect is contrary to what one would expect from a general cytotoxic substance.

Tumor promotion has been proposed to involve alterations in normal cell differentiation (8, 12, 25). Despite dramatic alterations in tissue organization, VYS cellular development and differentiaion were not affected, at least qualitatively, by TPA treatment.
Endothelial cells and erythroblastic cells, which differentiate over the 30-hr culture period, were present in TPA-treated tissues at the end of the culture. An important observation concerns TPA actions on 11.4 day conceptuses. These conceptuses have a complete yolk sac vascular system at the time of treatment, and controls undergo little further differentiation over the 30-hr culture period. Although TPA was less potent than in 10.4 day conceptuses, nevertheless, separation of the VYS cellular layers was complete by the end of the culture. An important observation concerns TPA in the 30-hr culture period, were present in TPA-treated tissues at the time of treatment, and at the end of the culture. From other studies in our laboratory, it seems that TPA can affect the development of mouse embryos in vivo. We have observed embryonic death and specific kidney abnormalities in mouse fetuses following maternal TPA administration in the organogenesis phase. Several reports have indicated the presence of serum factors that influence the potency and/or biological effects of TPA. Horowitz et al. (19) have identified a factor(s) in calf, human, monkey, and rat serum that inhibits the binding of a phorbol diester to rat embryo fibroblasts. This inhibitory factor(s) was not related to serum lipids binding the phorbol ester nonspecifically but, related to a high-molecular-weight protein which was not albumin. Nagasawa and Little (27) have emphasized the importance of a heat-labile serum component which dramatically alters the biological properties of TPA. All reports indicating the positive effect of TPA to induce sister chromatid exchanges utilized heat-inactivated serum (15, 22, 26, 27). However, when the serum was not heat inactivated, all results were negative (10, 14, 24, 38). Nagasawa and Little (27) speculate that the heat-labile serum factor could be superoxide dismutase, an enzyme with a suppressive effect on the TPA induction of sister chromatid exchanges that is lost when it is first heat treated at 55°C for 30 min.

We therefore have investigated the potential of serum manipulation to alter the effects of TPA. As seen in Table 3, this study identifies both a heat-sensitive serum factor(s) and a nonfilterable serum component(s) which alter the effects of TPA. Concerning the nonfilterable component, the pore size (0.45 μm) of the filter used is too large to specifically exclude high-molecular-weight macromolecules. Large proteins, however, can "bind" to membrane filters in a nonspecific fashion. We suggest that the membrane filter has retained a serum component that interacts with TPA. It will be interesting to determine if this component binds to TPA itself, thereby lowering the effective free concentration; occupies binding sites on target molecules (putative receptors) of TPA preventing its binding; directly or indirectly antagonizes the biological responses produced by TPA; or is effective by some other mechanism.

Consistent with the work of Nagasawa and Little (27), heat treatment of the culture serum effected the actions of TPA. One can speculate that heat treatment activated a factor(s) which is capable of potentiating TPA action. A more likely explanation is that heat treatment inactivated a factor(s) which can either metabolize TPA to inactive compounds or protect against the biological effects of TPA. Studies are presently being conducted to ascertain if the heat-sensitive factor(s) is superoxide dismutase or related enzyme systems.

In summary, the results reported in this paper indicate that TPA causes the abnormal progressive separation of the cellular layers that constitute the rat embryonic VYS. The effective concentrations and structure-activity relationships of a series of promoters suggest that this effect could be related to late-stage tumor promotion. Although the data indicate that this effect is not related to general cytotoxicity, cellular hyperplasia, or alteration in tissue differentiation, it is not known at this point if this effect is associated with: changes in DNA, RNA, or protein synthesis; induction of ornithine decarboxylase activity; alteration in hemoglobin content or synthesis (as a quantitative measure of cellular differentiation); or effects on the cell surface. We are currently investigating these possibilities and believe the rat VYS maintained in vitro may provide a good model system for the study of the actions of tumor promoters.

REFERENCES


Fig. 1. A, rat conceptuses 6 hr after treatment with 2.5 µl DMSO (control) or TPA (50 nM). Note the blebs or swellings caused by TPA treatment. B, rat conceptuses 28 hr after treatment with 2.5 µl DMSO (control) or TPA (50 nM). The control conceptus has a complete plexus of VYS vessels and functioning circulatory system. TPA treatment has caused the VYS to become abnormally smooth and avascular with no apparent circulation.

Fig. 2. A, B, DMSO-treated (2.5 µl) control conceptus (x 8) and VYS (x 100), respectively, 28 hr after treatment. Throughout the culture period, control VYSs are characterized by a tight association between the endodermal (ENDO) and mesodermal (MESO) layers with the development of blood vessels between them. C, D, TPA-treated (50 nM) conceptus (x 8) and VYS (x 100), respectively, 18 hr after treatment. The endodermal and mesodermal layers of the VYS are partially separated by TPA treatment. E, F, TPA-treated (50 nM) conceptus (x 8) and VYS (x 100), respectively, 28 hr after treatment. The endodermal and mesodermal layers of the VYS are completely separated, with the mesoderm located abnormally close to the amnion (AMN). H & E.
Tumor Promoter Actions on Rat Embryonic Development in Culture

Brian E. Huber and Nigel A. Brown

*Cancer Res* 1983;43:5544-5551.

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