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Interaction of Epidermal Growth Factor With Initiators and Complete Carcinogens in the C3H10T1/2 Cell Culture System

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Unlike 12-O-tetradecanoylphorbol-13-acetate, epidermal growth factor (EGF) could not promote the appearance of type III foci from initiated C3H10T1/2 cells. At appropriate concentrations, EGF induced the formation of type II colonies in the absence of any initiator. At higher concentrations, EGF suppressed the induction of both type II and type III colonies elicited by methylcholanthrene.

Key words: epidermal growth factor, 12-O-tetradecanoylphorbol-13-acetate, EGF, TPA, promotion, initiation, transformation

The two stages of carcinogenesis, initiation and promotion, have been demonstrated in the murine C3H10T1/2 cell culture model transformation system, with chemicals [1], ultraviolet light [2], or x-rays [3] as initiators and 12-O-tetradecanoylphorbol-13-acetate (TPA) as the promoter. Fisher et al [4] subsequently reported that the potent mitogen epidermal growth factor (EGF) could also promote radiation-induced cell transformation in the C3H10T1/2 system. We previously reported that EGF shortened the latency and increased the frequency of methylcholanthrene (MCA)-induced skin papillomas under conditions where the carcinogen alone induced some tumors [5]. We were, therefore, also interested in examining the interaction of EGF with initiators and with complete carcinogens in the C3H10T1/2 system. In this communication, we report the effect of EGF on C3H10T1/2 cell transformation (i) with initiators, (ii) by itself, and (iii) in the presence of a complete carcinogen.

MATERIALS AND METHODS

Two-stage transformation of C3H10T1/2 cells was assayed as described previously [1]. C3H10T1/2 cells (2,000 cells seeded per dish) were plated in 60-mm dishes and irradiated 24 hr later in the absence of medium. Cells were refed, and after 96

Abbreviations used: EGF, epidermal growth factor; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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hr, the medium was changed to fresh medium containing the indicated promoter test substance. Medium was changed twice weekly until the cells were confluent and once weekly thereafter. Promoter was added with each medium change. After 6 wk, the plates were washed, fixed with methanol, stained with Giemsa, and scored for transformed foci. Morphological criteria for type II and type III foci have been described previously [6]; representative colonies are illustrated in Figure 1.

TPA (Peter Borchert, Eden Prairie, MN) was dissolved in acetone. Final concentrations of acetone did not exceed 0.5%. Epidermal growth factor was purified by the procedure of Savage and Cohen [7]. The biological activity of TPA and EGF was confirmed in mitogenic assays, performed as described previously [8,9], on 3T3 cells.

RESULTS AND DISCUSSION

EGF Does Not Promote the Appearance of Type III Foci

In our initial experiments, we wished to determine whether EGF, like TPA, could promote the cellular transformation of initiated C3H10T1/2 cells. Our most

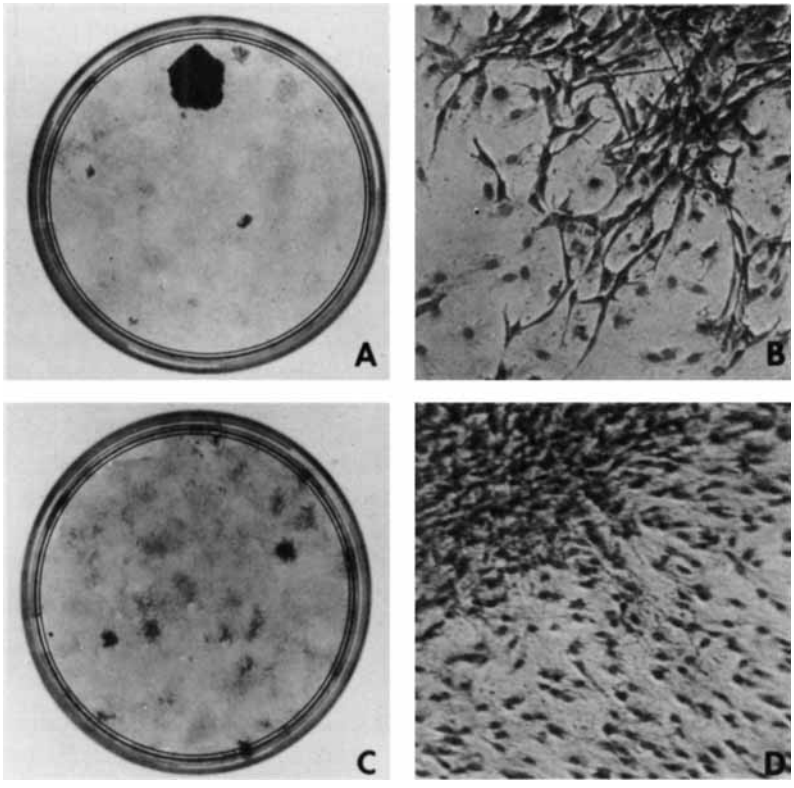

Fig. 1. Representative type II and type III colonies of C3H10T1/2 cells. Panel A shows a plate with a prominent type III focus. The photomicrograph of this type III colony, shown in panel B, demonstrates the polar fibroblastic multilayered nature of the colony, with an invasive edge. Panel C shows a plate with several type II foci. The photomicrograph in panel D illustrates the compact, piled-up appearance of these colonies, and the absence of an invasive edge.

extensive set of experiments was carried out with ultraviolet (UV) light as the initiator, since substantial transformation is not observed with UV irradiation alone [2]. In accordance with earlier studies [1,2], we initially scored only type III colonies as positive in this assay. In our experiment, TPA was, as previously reported, an active promoter (Table I), greatly increasing the number of type III colonies when applied after UV irradiation. However, EGF was unable to elicit an increase in type III foci after UV irradiation. It should be noted that only type III colonies were considered positive in the original descriptions of the C3H10T1/2 assay system for the two-stage transformation [1,2]. Our data differ from those of Fisher et al [4], who reported that EGF promoted the appearance of both type II and type III foci. We seen no enhancement of type III colonies, while they report that type III foci accounted for 70–90% of the transformed foci in their assay. While it is possible that this difference is simply the result of differences in evaluation of morphological criteria, the characteristics of type II and type III colonies are quite different [6] (see Fig. 1).

In separate experiments, EGF was also unable to increase the number of plates with type III foci when cells had previously been exposed to 0.1 μg/ml MCA (an initiating dose) or 0.25 μg/ml azacytidine. In contrast, TPA was able to promote the appearance of type III foci in experiments with these chemical initiators (Table II). The EGF concentration used in our experiments spanned the range active for EGF-induced mitogenesis [8,9], enzyme induction [10], and a variety of other biological responses.

EGF Alone Causes the Appearance of Type II Foci

In the course of the experiments described above, it appeared to us that EGF alone might have been causing some morphological changes in the C3H10T1/2

TABLE I. TPA, but Not EGF, Promotes the Appearance of Type III Colonies After UV Initiation*

UV (100 ergs/mm^2)	TPA (100 ng/ml)	EGF (ng/ml)	No. of dishes with type III foci/No. of dishes plated	Percent of dishes with type III foci
–	–	–	0/122	0.0
–	+	–	3/112	2.8
+	–	–	1/63	1.6
+	+	–	12/52	23.1
+	–	0.5	0/20	0
+	–	1.0	0/17	0
+	–	5.0	0/10	0
+	–	10.0	0/10	0
+	–	100.0	0/20	0
+	–	500.0	0/20	0
+	–	1,000.0	0/20	0

*The two-stage transformation assay is described in Materials and Methods. Data are expressed as the number of dishes with transformed foci divided by the total number of dishes and as percent of dishes with transformed foci. The transformed cells in a single dish are potentially motile; it is possible that multiple colonies in a single dish could arise from a single transformed cell. This presentation of the data, therefore, is the most conservative estimate of transformation events. Plating efficiency tests were performed by exposing 200–400 cells to similar treatments as those described for transformation, but fixing and counting colonies at 7–10 days. Plating efficiency for control cells was 21%; for cells treated with 100 ng/ml EGF (the highest concentration tested), 22%; for TPA treated cells, 15%; and for cells exposed to UV light, 14%.

TABLE II. TPA, but not EGF, Promotes the Appearance of Type III Colonies After Initiation by Azacytidine or Methylcholanthrene*

Experiment: initiator	Promoter	No. of dishes with type III foci/No. of dishes plated	Percent of dishes with type III foci
Experiment 1: azacytidine (0.25 μg/ml)			
	—	5/29	17.2
	TPA, 100 ng/ml	22/26	84.6
	EGF, 5 ng/ml	2/10	20
	EGF, 50 ng/ml	1/10	10
	EGF, 250 ng/ml	1/10	10
Experiment 2: methylcholanthrene (0.1 μg/ml)			
	—	2/45	4.4
	TPA, 100 ng/ml	6/49	12.2
	EGF, 5 ng/ml	0/10	0
	EGF, 10 ng/ml	0/10	0
	EGF, 50 ng/ml	0/20	0

*Plating efficiency for control cells in experiment 1 was 14.7%; with azacytidine, plating efficiency was 9.8%. Plating efficiency for control cells in experiment 2 was 17.7%; with methylcholanthrene, plating efficiency was 15.1%.

monolayers. To quantitate these effects and to eliminate the interaction with initiators, we exposed C3H10T1/2 cells to various concentrations of EGF and scored for type II and type III foci (Fig. 2). To our surprise, we observed induction of type II foci in the presence of EGF alone. No type III foci occurred in the presence of EGF. No type II or type III foci occurred in any of the 25 control plates for this experiment.

The induction of type II foci by EGF appears to follow a bell-shaped curve (Fig. 2), similar to a growth-promoting response reported for EGF [11] and for EGF induction of cellular adhesion [12]. To analyze the statistical significance of the decline in type II foci at higher EGF concentrations, we first tested the null hypothesis that there is no correlation for the 10- and 100-ng/ml values between the proportions of dishes with type II foci and the EGF concentration; the null hypothesis was rejected with a P value of 0.0015 (Yates corrected chi-square analysis). To use all the available data points, we tested for a linear trend for the ascending (1, 5, and 10 ng/ml) and descending (10, 50, and 100 ng/ml) portions of the curve in Figure 2. The null hypothesis (ie, that linear trends did not exist) was rejected in both cases ($P = 0.0024$ for the ascending limb, $P = 0.0015$ for the descending limb).

In contrast to our results, Fisher et al [4] reported that EGF alone was unable to elicit either type II or type III foci. Frazelle et al [13] have reported that, in some serum lots, TPA alone is able to cause both type II and type III foci. In examining data from a variety of experiments in which C3H10T1/2 cells were treated with TPA (100 ng/ml) alone, we find that 28% of the dishes had type II foci. Thus, we conclude that (i) EGF and TPA may both be able, in some serum lots, to cause focus formation in the absence of exogenous initiator, and (ii) the difference in the results of Fisher et al [4] and those presented here for induction of type II foci by EGF may well be similar to those differences observed for different serum lots [13] with TPA.

EGF Suppresses Focus Formation by Transforming Levels of MCA

In our final set of experiments, we investigated whether EGF could influence the transformation assay after cells had been exposed to a level of a complete

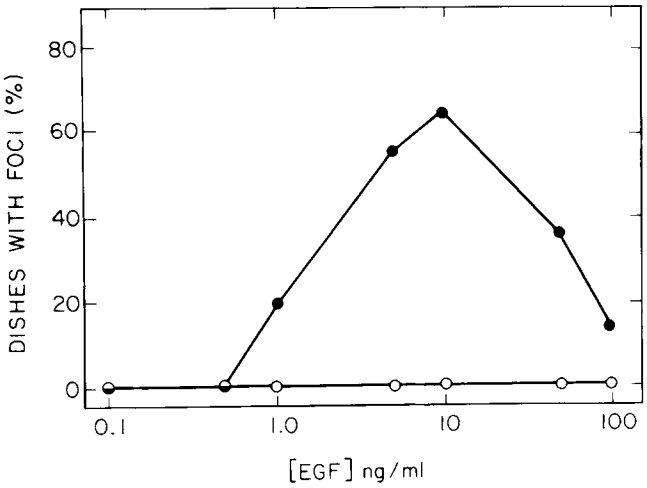

Fig. 2. Epidermal growth factor (EGF) alone induces type III but not type II foci. Cells were treated only with EGF, at the concentrations shown. No initiator was present. The transformation assay is similar to that described in the footnote to Table I. Twenty-five plates were analyzed at each EGF concentration. No type II or type III colonies were present on any of the 25 control plates. (●), type II colonies; (○), type III colonies.

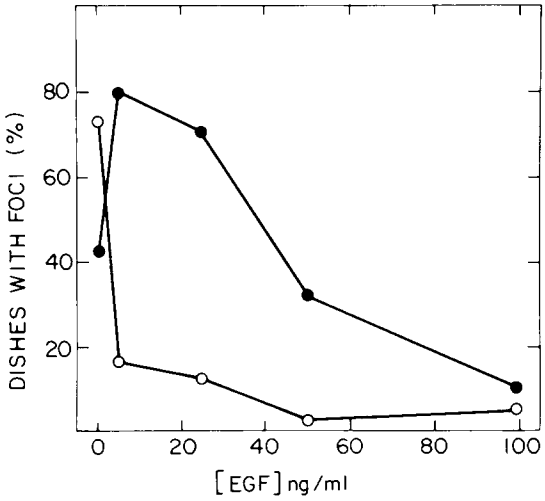

Fig. 3. Epidermal growth factor suppresses transformation induced by methylcholanthrene (MCA). Methylcholanthrene was dissolved in acetone. The final concentration of acetone in the medium of MCA treated cells was 0.5%. The concentration of MCA was 2.5 μg/ml. C3H10T1/2 cells (2,000) were plated in 60-mm dishes. After 24 hr, the medium was removed, and fresh medium containing MCA was applied. Twenty-four hours later, the medium containing MCA was removed, and the cells were fed with fresh medium without carcinogen. After an additional 72 hr, medium was once again changed to fresh medium containing the indicated concentration of EGF. The remainder of the transformation assay was carried out as described in the footnote to Table I. Thirty dishes were scored for each EGF concentration. Plating efficiency for control cells was 18%; plating efficiency for MCA treated cells was 14%. (●), type II colonies; (○), type III colonies.

carcinogen adequate to cause, by itself, the appearance of type III foci. A concentration of 2.5 μg/ml MCA should elicit a substantial number of transformants, without extensive toxicity [6]. In our experiment, MCA at 2.5 μg/ml induced type III foci in 73% of the experimental dishes (Fig. 3); 42% of the MCA treated dishes had type II foci. Epidermal growth factor at relatively low doses reduced the number of type III foci, an unexpected result. Moreover, at higher doses of EGF (100 ng/ml), the level of both type II and type III foci induced by MCA was dramatically suppressed. Thus, regardless of the relative tumor-inducing capacity of type II versus type III foci, 100 ng/ml EGF is able to reduce the transforming efficacy of MCA for C3H10T1/2 cells.

We have previously reported that EGF, when applied to mouse skin following a carcinogenic dose of MCA, shortened the latent period prior to tumor appearance and increased the frequency of tumors [5]. We expected to find a similar result for the C3H10T1/2 transformation assay, ie, an increase in the number of foci in the presence of EGF after exposure to transforming levels of a complete carcinogen. Instead, we found that the cell culture transformation system was affected in the opposite direction by EGF; transformation was suppressed by this growth factor. At present we do not have any clear idea why the cell culture and animal experiments differ in their interactions between EGF and MCA. We are currently determining the minimal time period during which EGF must be present to suppress transformation by MCA.

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