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SPECIFIC BINDING OF TRANSFORMING GROWTH FACTOR CORRELATES WITH PROMOTION OF ANCHORAGE INDEPENDENCE IN EGF RECEPTORLESS MOUSE JB6 CELLS

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SUMMARY

Tumor promoting phorbol diesters have previously been shown to irreversibly promote transformation to anchorage independence and tumorigenicity in mouse JB6 epidermal cells. This report demonstrates irreversible induction of anchorage independence in a clonal line of JB6 by epidermal growth factor (EGF) and by human transforming growth factor (TGF). TGF, but not EGF, promoted transformation in an EGF receptorless variant of JB6, R219. R219 cells specifically bind [^{125}I]-TGF but not [^{125}I]-EGF which indicates the existence of a specific receptor for TGF other than the EGF receptor.

INTRODUCTION

Transforming growth factors (TGF's) secreted by certain human tumor cells and similar to sarcoma growth factors (1,2) have been described by Todaro et al. (3). These factors interact with epidermal growth factor (EGF) receptors and induce nontransformed cell lines to grow in soft agar.

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It has been proposed that these TGF's exert transforming activity through binding to EGF receptors while binding of EGF to the same receptors results in a different nontransforming biological activity when assayed on nontransformed indicator cells (4,3).

Studies in our laboratory have shown that 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a potent tumor promoting agent irreversibly induces anchorage independence and tumorigenicity in JB6 mouse epidermal cells (5,6). Nonphorbol tumor promoters and EGF also promote transformation in JB6 cells (7). Recently we have described the derivation of clonal lines of JB6 cells which lack available EGF receptors (8,9,10). We report here that these EGF receptorless cells are irreversibly promotable to anchorage independence not only by TPA but also by a TGF preparation (3) from a human rhabdomyosarcoma line which showed EGF competing activity. Furthermore, [^{125}I]-TGF shows specific binding to the EGF receptorless cells which is not inhibited by EGF or phorbol dibutyrate.

METHODS AND MATERIALS

The cells used were derived from the TPA promotable JB6 mouse epidermal cells by nonselective cloning (JB6 Cl 41 and JB6 Cl 21) or by selection of JB6 Cl 41 cells for TPA resistance as described (7,8). Anchorage independent growth was measured in 0.33% agar and colonies $\geq$ 8 cells scored at 14 days (5). ^{125}I-EGF was obtained from KOR biochemicals. Unlabeled EGF was obtained from Collaborative Research, Waltham, Mass. Highly purified EGF was kindly supplied by Dr. Bruce Magun, University of Arizona. ^{125}I-TGF and unlabeled TGF from the A673 human rhabdomyosarcoma line (3) were kindly provided by Charlotte Fryling and Dr. George J. Todaro, National Cancer Institute, Frederick, Maryland.

^{125}I-EGF and ^{125}I-TGF binding were performed at 4^{o}C on confluent cultures in six or twelve well plates (Costar) according to the method of Magun et al. (11). Nonspecific binding was determined by measuring the

TABLE 1

INDUCTION OF ANCHORAGE INDEPENDENCE BY GROWTH FACTORS

Agent	Conc.	Anchorage Independence Response (Colonies/10^4 cells)
NONE		<1
TPA	10 ng/ml (1.6×10^{-8}M)	2739 ± 147
EGF	20 ng/ml[a]	1547 ± 50
	2 ng/ml[b]	1194 ± 42
TGF	5 g/ml	1517 ± 68

The JB6 Cl 41 mouse epidermal cell line was derived as described (7) and assayed for induction of anchorage independence after exposure of cells to inducer in 0.33% agar (5,7) containing 10% fetal calf serum. The TGF preparation used was the 20-23,000 m.w. peak material obtained from A673 cells after separation by Biogel P-100 chromatography and supplied by C. Fryling and Dr. G. J. Todaro (3).

[a]EGF from Collaborative Research. This partially purified EGF at 2 ng/ml produced little or no colony formation.

[b]Purified EGF supplied by Dr. Bruce Magun.

Results are expressed as the mean value for 2 experiments run in duplicate and the variability as one-half the range.

amount of radiolabeled ligand bound in the presence of 100-1,000 fold excess unlabeled ligand.

RESULTS

As reported previously (7), JB6 cells show clonal heterogeneity for the promotion of anchorage independence response to TPA as assayed by colony induction in 0.33% agar medium. JB6 clone 41 is sensitive to promotion of transformation by both TPA and EGF while other JB6 clones are resistant to both TPA and EGF (7). The promotion of anchorage independence response of JB6 Cl 41 cells to TPA, EGF and TGF from human A673 tumor cells is shown in Table 1. At 5 ug/ml, partially purified TGF containing

TABLE 2

IRREVERSIBLE INDUCTION OF ANCHORAGE INDEPENDENCE IN JB6 CELLS

Inducer	Cell Line	Soft Agar Colony Yield in the Absence of Inducer (Colonies/10^4 Cells)
TPA	T2194	2280
	T2197	1698
	T4114	1266
	T2192	1254
	T2193	1080
	T2199	648
	T2198	528
	T2195	192
	T2196	0
EGF	E3	1920
	E6	1800
	E9	1704
	E1	1032
	E8	534
	E13	456
	E7	420
	E11	48
TGF	G4119	4260
	G4121	2904
	G2192	2202
	G413	1968
	G2197	456
	G2193	324
	G4120	24

Colonies were picked from soft agar as described (5) after induction by the indicated inducer under conditions in which untreated JB6 Cl 21 or JB6 Cl 41 cells produced zero colonies (i.e. $<10^{-4}$). Cell lines were established, then assayed for growth in 0.33% agar in the absence of inducer as described (5).

20,000-23,000 M.W. material separated by Biogel P-100 chromatography, showed promoting activity similar to that produced by 2 ng/ml or 3.3×10^{-10}M purified EGF. This production of about 1500 soft agar colonies per 10^4 cells represents over half the level of activity shown by 1.6×10^{-8}M TPA, a near saturating concentration for TPA induction of anchorage independence (5). Colony size was typically 50-200 cells per colony for colonies induced by TPA and 20-100 cells for colonies induced by EGF and TGF.

Table 2 shows that the majority of the colonies produced from JB6 Cl 41 or Cl 21 by either TPA, EGF, or TGF gave rise to transformed cell lines

TABLE 3

R219 CELLS LACK EGF RECEPTORS

Cell Line	[^{125}I]-EGF Binding [^{125}I]-EGF Alone (DPM/well)	+Excess EGF (DPM/well)	Specific Binding (DPM 10^6 Cells)
JB6 Cl 41	1035	41	3610 ± 118
R219	39	33	17 ± 20

Binding was determined after incubation with 0.2 ng/ml [^{125}I]-EGF (0.02 uc/ml per well containing 2-4 x 10^5 cells) for 4 hours at 4°C in 1% bovine serum albumin as described (11). R219 cells were clonally derived after selection of JB6 Cl 41 cells for resistance to plateau density mitogenic stimulation by TPA (8). Results are given as the mean DPM/10^6 cells for duplicate wells. The experiment was repeated three times.

which formed colonies in agar in the absence of inducer. Under the conditions of this assay (serum lot, etc.), approximately 70 to 75% of the induced lines showed irreversible acquisition of anchorage independence. Assay of these cell lines 2 to 6 passages later confirmed the phenotypes shown in Table 2.

We have recently reported on the isolation of a series of clonal derivatives of JB6 Cl 41 which were resistant to plateau density mitogenic stimulation by TPA (8,9). As shown in Table 3, one such TPA-mitogen resistant cell line, R219, showed no specific binding of [^{125}I]-EGF. Scatchard analysis of the parent JB6 Cl 41 line showed 60,000 binding sites per cell (10). Table 4 shows that this EGF receptorless cell line is promotable to anchorage independence by TPA and TGF but not by EGF. The colony yield produced by exposure of R219 to the more purified EGF was about 2% as high as that observed for EGF on Cl 41. The colony inducing activity for TGF on R219 was similar to that found for the parent JB6 Cl 41 (Table 1) and for TPA acting on R219 (Table 4).

This suggested the possibility that these cells may contain in addition to EGF receptors another class of binding sites for TGF through

TABLE 4

TGF PROMOTES ANCHORAGE INDEPENDENCE IN R219 CELLS

Agent	Conc.	Anchorage Independence Response (Colonies/10^4 cells)
NONE		42 ± 30
TPA	10 ng/ml (1.6×10^{-8} M)	2250 ± 401
EGF	20 ng/ml[a]	270 ± 144
	2 ng/ml[b]	84 ± 16
TGF	5 ug/ml	1950 ± 80

Assays for promotion of anchorage independence and the derivation of R219 were described in the legends to Tables 1 and 3. Results are expressed as the mean value for 2 experiments run in duplicate and the variability as one-half the range.

which the TGF exerts its biological activity for promotion of transformation.

TGF was isolated after carboxymethylcellulose chromatography from an EGF receptor competing peak having agar colony inducing activity (3, Fig 5), then [^{125}I]-labeled. Table 5 shows the binding of [^{125}I]-TGF to R219 cells and the JB6 Cl 41 parent line. The specific [^{125}I]-TGF binding competable by excess unlabeled TGF accounted for about 60% and 35% of the total for R219 and Cl 41 respectively. EGF competable binding accounted for little or none of the total. [^{125}I]-TGF binding was also reduced by only 2 to 8% in the presence of excess amounts of the tumor promoter phorbol dibutyrate which has been shown to have specific cellular binding sites (12).

DISCUSSION

These results have shown that specific binding of labeled transforming growth factor occurs in clonal lines of JB6 in which TGF irreversibly promotes anchorage independence. One such cell line, R219, was EGF receptorless, suggesting that although this human A673 TGF does bind to EGF

TABLE 5

[^{125}I]-TGF BINDS SPECIFICALLY TO R219 CELLS

Cell Line	[^{125}I]-TGF Binding (DPM/well)				[^{125}I]-TGF Specifically Bound (DPM/10^6 cells)		
	[^{125}I]-TGF only	+TGF	+EGF	+PDBu	VS TGF	VS EGF	VS PDBu
Expt. 1							
R219	3079	1257			1400 ± 62		
JB6 Cl 41	1773	1137			535 ± 25		
Expt. 2							
R219	1394		1501[a]	1302	0		120 + 37
			1339[b]			72 ± 34	
JB6 Cl 41	870		960[a]	846	0		27 ± 30
			945[b]		0		

[^{125}I]TGF from A673 cells was obtained from the EGF receptor competing peak of soft agar colony inducing activity after carboxymethyl cellulose chromatography (3, Fig. 5), iodinated as described for EGF(3), and kindly provided by Charlotte Fryling and Dr. George Todaro. [a,b] was that used for Table 1. [^{125}I]-TGF binding at a concentration of ^{125}I-TGF which gave 50% competition with EGF for binding was assayed at 4°C with or without excess unlabeled TGF (100-fold), EGF (500 ng/ml Collaborative; 50 ng/ml Magun's) or phorbol dibutyrate (PDBu, 30 uM, 2 ug/ml). Results were expressed as the mean for duplicate wells (35 mm in Expt. 1; 22 mm in Expt. 2).

receptors in a competition assay (3), the EGF receptor binding is not necessary for the transforming activity of this TGF preparation. A similar conclusion was suggested earlier for sarcoma growth factor (SGF) in a report by Pruss, Herschman and Klement (13). Murine and feline retroviruses transform 3T3 or other cells by inducing the producion of transforming polypeptides called sarcoma growth factors which bind to EGF receptors; the binding of SGF to EGF receptors has been postulated to mediate SGF transforming activity and to account for the low levels of available EGF receptors found in SGF-producing cells (1,2,4). Pruss et al. (13) observed that EGF receptorless variants of 3T3 cells were susceptible to transformation by Kirsten sarcoma virus thus suggesting that SGF must act through receptors other than EGF receptors.

More recently TGF's which show similar biological activities to SGF have been isolated from a number of human and rodent tumor cells or the

medium in which they were grown (4,14,15). Some of these TGF's, for example factors described recently by Moses et al. (15) isolated from chemically transformed mouse cells, showed potent stimulation of colony formation in soft agar by nontransformed cells but no EGF receptor competing activity. Todaro et al. (3) have described a minor peak of agar colony inducing activity from A673 cells which lacks EGF receptor competing activity. The A673 TGF used on these studies for determining biological activity contained both this noncompeting material and the predominant species of EGF competing material. The [^{125}I]-TGF used for binding studies was the predominant EGF receptor competing peak (3).

That [^{125}I]-TGF shows specific binding to the R219 EGF receptorless cells suggests that they contain a specific membrane receptor for TGF to which neither EGF nor phorbol diesters can bind. These cells should, therefore, be useful for TGF receptor purification.

It has been postulated that binding of TGF and EGF (which induces little or no agar colony formation by 3T3 or NRK cells) to the same receptor leads to different biological consequences. It is clear from the results reported here that there is no need to invoke such a mechanism since the differential activities may be explainable by binding to 2 different receptors.

Mouse JB6 cells show two noteworthy differences from 3T3 and NRK cells in their responses to growth factors. The first is that TGF but not EGF induces agar colony formation by the fibroblast indicator cells whereas both TGF and EGF induce anchorage independent growth by JB6 cells. The second is that the TGF induction of anchorage independent growth is reversible for 3T3 and NRK cells but irreversible for JB6 cells. Since the JB6 cells are nontransformed but irreversibly promotable to tumor cell phenotype by tumor promoters (5) while 3T3 and NRK cells are not (not shown), the 3T3 and NRK cells may represent an early preneoplastic stage and the JB6 cells a late preneoplastic stage of progression. The TGF may induce 2

steps in the process of preneoplastic progression, one of which is also shared by EGF. Late preneoplastic cells may require only the common step for induction of anchorage independence while early preneoplastic cells may require both. The JB6 cell phenotype differs from that of 3T3 and NRK cells in not requiring continuous exposure to SGF or TGF (4,16) to maintain the transformed phenotype once induced. The possibility that transformed JB6 cells are producers of transforming growth factors appears unlikely since neither assays of conditioned medium nor mixing experiments suggested this (Colburn et al., unpublished). Current studies are focused on elucidating the critical biochemical changes induced by TGF or EGF or TPA when they act as promoters of transformation in JB6 cells.

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