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CHANGES IN EPIDERMAL GROWTH FACTOR RECEPTORS ASSOCIATED WITH ADENOVIRUS TRANSFORMATION, CHEMICAL CARCINOGEN TRANSFORMATION AND EXPOSURE TO A PHORBOL ESTER TUMOR PROMOTER

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SUMMARY: Transformation of rat embryo cells by wild type or a mutant (H5ts125) of human adenovirus type 5 results in >97% reduction in binding of epidermal growth factor to cell surface receptors. A 26 to 89% reduction in binding was observed in clones of NIH-3T3 cells transformed by benzo(a)pyrene (BP) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Chemically or spontaneously transformed rat liver epithelial cell lines and a rat hepatoma cell line also displayed low binding. The reduction of epidermal growth factor binding does not correlate quantitatively with expression of other markers of transformation. Brief exposure to a phorbol ester tumor promoter reduced epidermal growth factor binding in all of these cell types.

INTRODUCTION

Recent studies indicate that the potent tumor promoter TPA inhibits the binding of EGF to cell surface receptors in cultures from a variety of species and cell types (1-4). This effect is exerted by nanomolar concentrations of TPA, occurs within minutes after addition of the compound, correlates with the tumor promoting activity on mouse skin of a series of phorbol esters, and appears to be specific for EGF receptors (1-4). The effect may be related to a number of other membrane changes induced by the phorbol ester tumor promoters (for review see refs. 2,5,6,7). It is also likely that the membrane changes induced by the phorbol esters may underlie their highly pleiotropic effects on cells (for review see refs. 5 and 7).

Other recent studies suggest that changes in the EGF effector system may be important not only during the carcinogenic process but also for maintenance

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Abbreviations used: TPA, 12-O-tetradecanoyl-phorbol-13-acetate; EGF, epidermal growth factor; wt Ad5, wild type human adenovirus type 5; H5ts125, temperature sensitive mutant of Ad5; BP, benzo(a)pyrene; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; DMBA, 7,12-dimethylbenz(a)anthracene; AAAF, N-acetoxy-2-acetylaminofluorene; FBS, fetal bovine serum; MEM-10, Eagle's minimum essential medium with 10% FBS; DMEM-10, Dulbecco's modified MEM with 10% FBS; F12-10, Ham's F12 medium with 10% FBS; RE, rat embryo; ts, temperature-sensitive.

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of the tumor cell phenotype (8-12). EGF-receptor binding is reduced in 3T3 cells transformed by murine or feline sarcoma viruses (8-10). This appears to be due to the synthesis by these transformed cells of a polypeptide growth factor, termed sarcoma growth factor, which occupies the EGF receptors and can alter the phenotype of certain normal cells (8-11). Some, but not all carcinogen-transformed embryo fibroblast cultures also have reduced EGF receptors (10,12). On the other hand, cells infected with nontransforming RNA viruses or transformed by polyoma or SV40 viruses did not have a decrease in EGF receptors (10,12).

The endogenous production of various growth factors by tumor cells may be a fairly general phenomenon (8-11), since it even occurs in plant tumors (24). In this paper, we describe the effects on EGF receptor binding of: a) transformation of secondary rat embryo cells by wild type (wt Ad5) and a mutant (H5ts125) of human adenovirus type 5; b) transformation of the NIH-3T3 fibroblast cell line by chemical carcinogens; and c) neoplastic transformation of epithelial cells by chemical carcinogens. The effects of TPA on EGF binding by these various types of cells are also described.

MATERIALS AND METHODS

Cells and Media: RE cells cultures (13) were grown in DMEM-10. Ad-C6-L is a cloned population of transformed cells derived from 2^0RE cells infected with human adenovirus type 5 (wt Ad5), passaged >30 times since transformation (14). Ad-A18-E (passaged <10 times) and Ad-E7-L (passaged >30 times) are cloned populations of transformed cells derived from 2^0RE cells infected with H5ts125 a mutant of human adenovirus type 5 (15,16). BP-Ad-E11-L (passaged >30 times) is a cloned population of transformed cells derived from 2^0RE that were pretreated with benzo(a)-pyrene (BP) and then infected with H5ts125 (15-17). BP-Ad-E11-A-1-11 is an agar derived clone of BP-Ad-E11-L cells which is not responsive to TPA enhanced growth in agar (17,18). DMBA-Ad-B8-L and DMBA-Ad-C5-E are cloned populations of transformed cells derived from 2^0RE that were pretreated with DMBA and then infected with H5ts125 (15-17). All of these transformants were grown in low Ca^{2+} (0.1 mM Ca^{2+}) DMEM with 7.5% FBS.

A clone NIH-3T3 cells (provided by T. Kakunaga) with a low spontaneous transformation frequency was exposed to 1μg/ml BP or 0.1μg/ml MNNG for 24 hours in MEM-10. After 24 hours, the carcinogen containing media were removed, the plates were washed twice with MEM-10. The cultures were then maintained for 4 to 6 weeks in MEM-10 containing 10ng/ml TPA with weekly media changes. Transformed foci, characterized by a lack of contact-inhibition, were then isolated from separate plates and were maintained as separate cell lines in MEM-10.

The rat liver epithelial cell line, K22; a tumorigenic derivative obtained by *in vitro* transformation with AAAF, W8, and a ts mutant of W8, TS 223, have been previously described (20,21). The HTC cell line was isolated from a carcinogen induced rat hepatoma (20). The IAR 27 and IAR 20-PCI epithelial cell lines were derived from normal 10-week old BD VI rats (22), provided by R. Montesano. They were not tumorigenic after 24 weeks but were after 60 weeks in culture. K22 and IAR cells were grown in F12-10. W8, TS 223 and HTC cells were grown in DMEM-10. All cell cultures were grown in a 5% CO_2 humidifier air incubator at either 36^0 or 40^0C.

EGF Binding Assay: ^{125}I-EGF binding was determined as previously described (1,2). Cells were used in logarithmic phase of growth. Assays were done in duplicate and the values given are mean values. Duplicates agreed within 5%.

RESULTS

Binding of EGF to Normal and Adenovirus-Transformed Rat Embryo Cells

Transformation of 2^0RE cells by wt Ad5 virus resulted in greater than a 70 fold reduction in binding of ^{125}I-EGF (Table 1). A similar marked reduction in EGF receptors was observed in cells transformed by the H5ts125 mutant of Ad5, which is temperature-sensitive with respect to adenovirus DNA replication (Table 1) Transformed clones derived from 2^0RE that were pretreated with the initiating chemical carcinogens BP or DMBA prior to infection with H5ts125 had a reduction in EGF receptors which was similar to that found with transformed clones obtained with the H5ts125 virus alone (Table 1). Carcinogen pretreatment has been found to increase the transformation frequency (15) as well as the temporal acquisition of anchorage-independent growth (14, 16-18) in H5ts125 infected 2^0RE. The addition of TPA to the normal 2^0RE and to all of the wt Ad5 or H5ts125 transformed cells resulted in a 59 to 82% reduction in ^{125}I-EGF binding when compared to parallel untreated cultures, but TPA did not reduce the level of binding in 2^0RE cells to that of the adenovirus transformed clones (Table 1).

Binding of EGF to Normal and Chemically Transformed 3T3 Cells

The effects of transformation of NIH-3T3 mouse embryo fibroblasts by BP or MNNG on ^{125}I-EGF receptor binding are shown in Table 2. Although there were considerable variations, all 4 clones of BP transformed cells and both of the MNNG transformed clones had lower EGF binding than the untransformed 3T3 cells. The decrease varied from 26 to 89%, and thus was not as profound as that seen with

Table 1: Binding of Epidermal Growth Factor (EGF) to Adenovirus Type 5 Transformed Rat Embryo Cells.

Cell Type*	^{125}I-EGF bound/10^6 cells* -TPA	+TPA	TPA Inhibition (% of Control)
Normal:			
Secondary Rat Embryo	3259	1342	41
Adenovirus-Transformed:			
Ad-C6-L (wt Ad5)	45	8	18
Ad-A18-E (H5ts125)	30	13	43
Ad-E7-L (H5ts125)	25	10	40
BP-Pretreated, Adenovirus-Transformed:			
BP-Ad-E11-L (H5ts125)	34	13	38
BP-Ad-E11-A-11 (H5ts125)	25	10	40
DMBA-Pretreated, Adenovirus-Transformed:			
DMBA-Ad-B8-L (H5ts125)	34	12	35
DMBA-Ad-C5-E (H5ts125)	80	19	24

*The sources of the cell lines are described in "Materials and Methods" and Refs. 15 and 16. Later passage, >30, clones are referred to as L, and earlier passage, <10, clones are referred to as E. EGF binding assays were performed as described in References 1 and 2. The total ^{125}I-EGF used per assay was 56,720 cpm.(specific activity 43μCi/μg).

Table 2: Binding of ^{125}I-EGF to Normal and Chemically Transformed NIH-3T3 Cells

Cell Type	Transforming Agent	^{125}I-EGF bound (cpm/10^6 cells) -TPA	+TPA	TPA Inhibition (% of Control)
3T3	None	526	126	24
BP 2A	BP	60	37	62
BP 1	BP	221	47	21
BP b	BP	147	34	23
BP c	BP	139	18	13
M b	MNNG	282	39	14
M e	MNNG	391	23	6

The sources of the normal 3T3 cells and of the BP and MNNG transformed cells are given in "Materials and Methods". The EGF binding assay was performed as described in Refs. 1 and 2. The total ^{125}I-EGF used per assay was 32,000 cpm (specific activity 36μCi/μg).

adenovirus transformed 2^0RE cells (Table 1). The addition of TPA to normal 3T3 and to all of the transformants led to significant reductions in ^{125}I-EGF binding (Table 2).

The reduction in EGF binding in 3T3 cells associated with MNNG transformation appears to be due to transformation and not simply MNNG mutagenesis since we found that when HeLa cells were treated with MNNG, and either 6-thioguanine, 5-bromodeoxyuridine, concanavalin A or oubain resistant mutants were selected, these mutants had the same binding of ^{125}I-EGF as the parental line of HeLa cells (unpublished studies).

Binding of EGF to Various Types of Rat Liver Cell Cultures

When compared to the normal rat liver epithelial cell line K22, the AAAF transformed rat liver cell line W8 and a temperature sensitive derivative of W8,

Table 3: Binding of ^{125}I-EGF to Rat Liver Cultures

Cell Type	Description	Growth Temperature	^{125}I-EGF bound (cpm/10^6 Cells) -TPA	+TPA	TPA Inhibition (% of Control)
Experiment 1:					
K22	Normal Cell Line	36^0	636	115	18
W8	AAAF Transformed	36^0	132	15	11
W8	AAAF Transformed	40^0	127	43	34
TS 223	Temperature-Sensitive Mutant of W8	36^0	48	12	25
TS 223	Temperature-Sensitive Mutant of W8	40^0	50	29	58
HTC	Rat Hepatoma	36^0	20	19	95
Experiment 2:					
IAR 27, 24 Wks.	Nontumorigenic	37^0	237	27	11
IAR 27, 60 Wks.	Tumorigenic	37^0	72	14	19
IAR 20-PCI, 24 Wks. -	Nontumorigenic	37^0	680	129	19
IAR 20-PCI, 60 Wks. -	Tumorigenic	37^0	233	53	23

For a description of these cell cultures see "Materials and Methods" and Refs. 20-22. Cells were grown at the indicated temperature and then assayed at 37^0C for ^{125}I-EGF binding as described in Refs. 1 and 2. Each assay contained 50,425 cpm of ^{125}I-EGF.

TS 223, displayed a marked reduction (79-92%) in EGF binding (Table 3). Growth of W8 or TS 223, at either 36^0 or 40^{0}C did not affect the extent of EGF binding, even though growth of TS 223 at 40^{0}C causes partial reversion of its phenotype towards that of normal liver epithelial cells (20,21,25). The tumorigenic rat hepatoma cell line, HTC, had an extremely low level of EGF binding. Both of the epithelial cell lines established from normal rat liver, IAR 27 and IAR 20-PCI, showed a reduction in ^{125}I-EGF binding when after serial passages in vitro they underwent spontaneous transformation and became tumorigenic (Table 3). TPA induced a reduction in the binding of EGF to both the normal and transformed rat liver epithelial cells, although the magnitude of the reduction in some of the transformed cells was small due to the already low binding capacity of these cells.

DISCUSSION

The above results demonstrate that transformation of RE cells by human adenovirus type 5 or the H5ts125 mutant of this virus, causes a striking decrease in EGF-receptor binding. Pretreatment of RE cells with BP or DMBA prior to virus infection increases the frequency of transformation by H5ts125 (14,15). With carcinogen pretreatment the transformants may also acquire the ability to grow in agar after fewer subpassages (16) and display differences in their cell surface glycoproteins (26). However, no differences were seen in the extent of reduction of EGF binding between cells transformed by virus alone or the combined chemical-viral treatment (Table 1). BP-Ad-E11-A-1-11 has a 7 fold higher cloning efficiency in agar than the parental clone BP-Ad-E11-L (17,18). Whereas, TPA stimulates the growth in agar of BP-Ad-E11-L, this is not the case with BP-Ad-E11-A-1-11 (17,18). Yet both of these clones had low, and approximately the same levels of EGF binding, and in both, TPA further depressed EGF binding (Table 1). Thus, there is no simple correlation between EGF binding, cloning efficiency in agar and response to TPA.

The mechanism by which adenovirus transformation of RE cells causes a reduction in EGF binding is not presently known. It could involve production of a polypeptide growth factor similar to EGF. Alternatively, it could reflect alterations in cell surface membranes that influence the function of EGF receptors. Evidence has been presented that TPA acts through the latter mechanism (2). Despite the reduced levels of EGF binding of adenovirus transformed RE cells, some of these cells do respond to exogenous EGF by enhanced growth in agar (18). This is consistent with evidence that only a small fraction of EGF receptors are involved in physiologic responses (27). We must emphasize that at the present time we have no evidence that the reduction in EGF binding seen in the present study is critical to the mechanism of adenovirus transformation.

Transformation of NIH-3T3 mouse embryo fibroblasts by chemical carcinogens (Table 2), and carcinogen or spontaneous transformation of rat liver epithelial cells (Table 3), also resulted in a reduction in EGF binding. The degree of reduction was variable and generally not as great as observed in the adenovirus transformants. A correlation between tumorigenicity and reduction in EGF receptor binding was observed in two independently isolated rat liver epithelial cell lines (Table 3). With TS 223, EGF binding was low at both 36^0 and 40^{0}C, despite the fact that at 40^{0}C these cells have a more normal phenotype (20,21,25) and are less invasive on the chick chorioallantoic membrane (28). A lack of correlation between EGF binding and expression of certain *in vitro* markers of transformation or tumorigenicity has also been demonstrated in BP transformed Syrian Harmster embryo fibroblasts (12). Thus, as with the adenovirus transformed cells, it appears that maintenance of the transformed phenotype is due to a complex mechanism that involves more than the functional state of EGF receptors.

The present studies extend previous evidence (1-4) that TPA is a potent inhibitor of EGF binding in fibroblast and epithelial cell systems, both normal and transformed. The phenomenon is, therefore, a general one although its mechanism and significance with respect to tumor promotion remain to be elucidated.

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