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Cell Biology

Variants of 3T3 cells lacking mitogenic response to epidermal growth factor

(receptors/mitogens/colchicine selection)

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ABSTRACT **We have previously demonstrated that epidermal growth factor (EGF) can serve as a potent mitogen for 3T3 cells. We have now selected variant 3T3 cell lines unable to respond to EGF, in order to define cellular events unique to the EGF response and to distinguish which of these events are necessary and which are merely correlative to mitogenesis. By simultaneously treating cells with EGF and colchicine, we eliminated those cells stimulated by EGF to enter mitosis. Of the eight clonal EGF nonresponder variants selected by this procedure, none retains a functional EGF receptor. The EGF nonresponsive variant lines still retain the ability to respond to other mitogens.**

Epidermal growth factor (EGF) is a low molecular weight (6045) polypeptide isolated from the submaxillary glands of adult male mice (1). It is a potent stimulator of DNA synthesis and cell division in various primary culture systems (2–6). Recently, we have shown that EGF at concentrations between 10^{-10} and 10^{-9} M initiates cell division in nondividing 3T3 cells (7).

EGF-induced division of 3T3 cells provides a useful culture system for studying the way mitogens stimulate cell division. The identification and understanding of the events occurring in the regulation of cell proliferation has been a major problem in cell biology. The continuous nature of the 3T3 cell line lends itself to the use of a genetic approach to these problems. Selection of variant 3T3 cell lines unable to respond to EGF should help to identify events stimulated by EGF that differ from those initiated by other mitogens. Equally as important, mitogen nonresponsive variants will help to distinguish the necessary steps from those which are merely correlated with the EGF mitogenic response. This variant approach is not possible with a primary culture system; it can only be used with a continuous clonal cell line such as 3T3 which displays growth control in culture.

We have cloned eight variant lines that no longer have an EGF proliferative response, utilizing a selection procedure that combines EGF stimulation of cell division of confluent stationary phase 3T3 cells with colchicine arrest of the resultant mitotic cells. All eight EGF nonresponsive variants lack a functional EGF receptor. All the variants retain response to at least one known 3T3 mitogen, e.g., phorbol myristate acetate (PMA), prostaglandin F2α (PGF2α), or insulin. Our variants lacking a functional EGF receptor have been used to identify the EGF receptor on 3T3 cells (8) and to examine early mitogenic events that require the binding of EGF to its cell surface receptor (A. Aharonov, I. Vlodavsky, R. M. Pruss, C. F. Fox, and H. R. Herschman, unpublished data).

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MATERIALS AND METHODS

EGF Purification. EGF was purified either as described (7) or by the method of Savage and Cohen (1).

Reagents. Colchicine was from Sigma and $Na^{125}I$ was from New England Nuclear. PGF2α was provided by John E. Pike, Upjohn. PMA, from Consolidated Midland Corp., Brewster, NY, was stored as 1 mg/ml of solution in absolute ethanol at −20° until used. Insulin (24 international units/mg) was from Sigma, culture medium was from Gibco, and fetal calf serum was from Reheis.

Cells. Swiss 3T3 cells were grown as described (7).

^{125}I-Labeled EGF. EGF was iodinated by the method of Hunter and Greenwood (9). Briefly, 5–30 μg of EGF and 0.1 ml of 1 M potassium phosphate buffer (pH 7.5) were added to the vial containing 2 mCi of $Na^{125}I$. The reaction was initiated at 4° by adding 10–20 μl of chloramine T (10 mg/ml). After 40–60 sec, 10–20 μl of sodium metabisulfite (20 mg/ml) and 0.1 ml of potassium iodide (30 mg/ml) were added to terminate the reaction. Bovine serum albumin was added as a protein carrier, and iodinated EGF was separated on a Sephadex G-25 column. Specific activities were generally 1 Ci/μmol.

^{125}I-Labeled EGF Binding Assay. We used an adaptation of previously reported ^{125}I-labeled EGF binding assays (10, 11) to measure EGF receptors. Confluent 35-mm culture dishes were placed on ice and washed twice with ice-cold assay buffer [saline A (12) containing 0.1% bovine serum albumin]. Assay buffer (1.0 ml) was added, followed by ^{125}I-labeled EGF. After incubation for 90 min on ice, the plates were washed six times with ice-cold assay buffer. The cells were solubilized with 1.0 ml of 0.5 M NaOH, and radioactivity was determined in 0.5 ml of the solution in a Beckman Biogamma γ-spectrometer. To measure "nonspecific" binding, we added 10-fold excess of unlabeled EGF to dishes 10 min prior to the addition of the ^{125}I-labeled EGF. All assays were performed in duplicate. Nonspecific binding ranged from 5 to 15%.

Cloning. Cells were diluted with culture medium to a final concentration of less than one cell per ml. The cell suspension (0.1 ml) was added to each well of a Falcon microtest II plate. The following day, wells were scored for single cells.

Cell Counts. Cells were removed from the culture plates for counting as described (7). Cells were counted on duplicate plates using an Electrozone/Celloscope model 112TH (Particle Data Inc., Elmhurst, IL).

RESULTS

Selection of EGF Nonresponder Variants. Our selection procedure is a modification of the procedure used by Vogel *et*

Abbreviations: EGF; epidermal growth factor; PMA; phorbol myristate acetate; PGF2α; prostaglandin F2α.

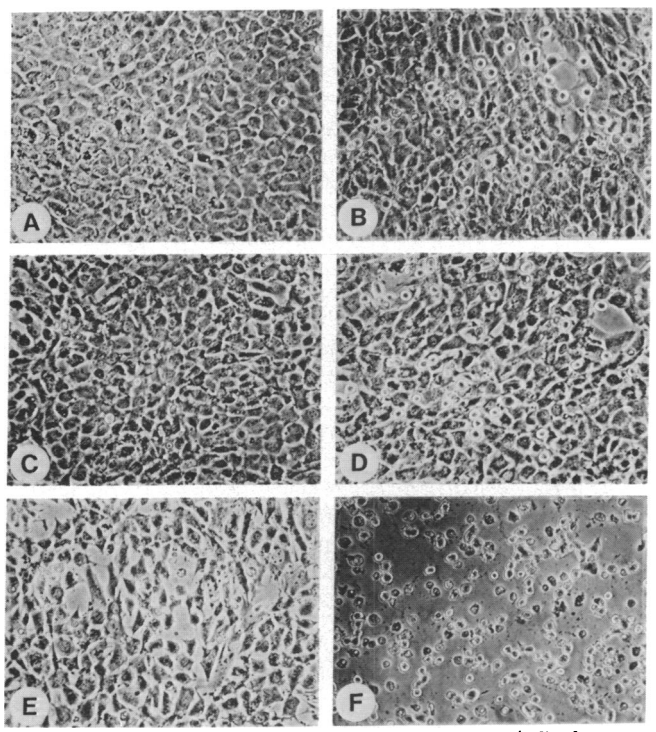

FIG. 1. 3T3 cells treated with colchicine (1.5 μg/ml) alone or colchicine and EGF (10 ng/ml). Cells treated with colchicine alone for (*A*) 1 day, (*C*) 2 days, and (*E*) 3 days. Cells treated with colchicine and EGF for (*B*) 1 day, (*D*) 2 days, and (*F*) 3 days.

al. (13) to isolate "flat" revertants of 3T3 cells transformed by simian virus 40. Cells (8×10^5) were plated in 10 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum in 100-mm Falcon tissue culture dishes, grown to confluence, and fed with fresh medium 1 day prior to simultaneous treatment with EGF (10 ng/ml) and colchicine (1.5 μg/ml). This concentration of colchicine blocks 3T3 cell division with a minimal effect on cell viability (data not shown). After 4 days, mitotic cells were washed off the plate with a stream of medium. The remaining cells were grown to confluence and passed 1:10. An illustration of the selection procedure is shown in Fig. 1. After three cycles, the cell lines were tested for their proliferative response to EGF as described (7). Those plates with a reduced EGF response were chosen for two more rounds of selection. Cell lines were cloned from populations with significantly decreased response to EGF (60% or less). Of 22 clonal cell lines, 8 showed no stimulation of cell division by EGF at either 10 or 100 ng/ml. An example of the response of 3T3 and one of the nonresponder variants (NR-6) is shown in Fig. 2. In no case did 100 ng/ml stimulate cells differently from 10 ng/ml. The cells selected by this procedure do not appear to be colchicine "resistant". The growth of both 3T3 cells and NR-6 and NR-7 variants is inhibited by 1.5 μg of colchicine per ml (data not shown).

Binding of ^{125}I-Labeled EGF to Cell Lines. Prior to testing the 3T3 and nonresponder variant cell lines for the ability to bind ^{125}I-labeled EGF, we tested the biological activity of the labeled EGF by measuring its ability to stimulate cell division. ^{125}I-labeled EGF (1.2 Ci/μmol) and unlabeled EGF were used to stimulate cell division in a dose-response experiment (7). Stimulation of growth by labeled and unlabeled EGF was identical (data not shown). Therefore, labeling EGF with ^{125}I did not affect the biological activity of the growth factor.

All eight nonresponder variant cell lines were tested for binding of ^{125}I-labeled EGF. None had measurable EGF receptor, even at concentrations of labeled EGF in 10-fold excess of the concentration necessary for maximal binding in the parent 3T3 cell line. The results of a binding assay on 3T3 and three of the EGF nonresponders (NR-1, NR-6, and NR-8) are shown in Fig. 3.

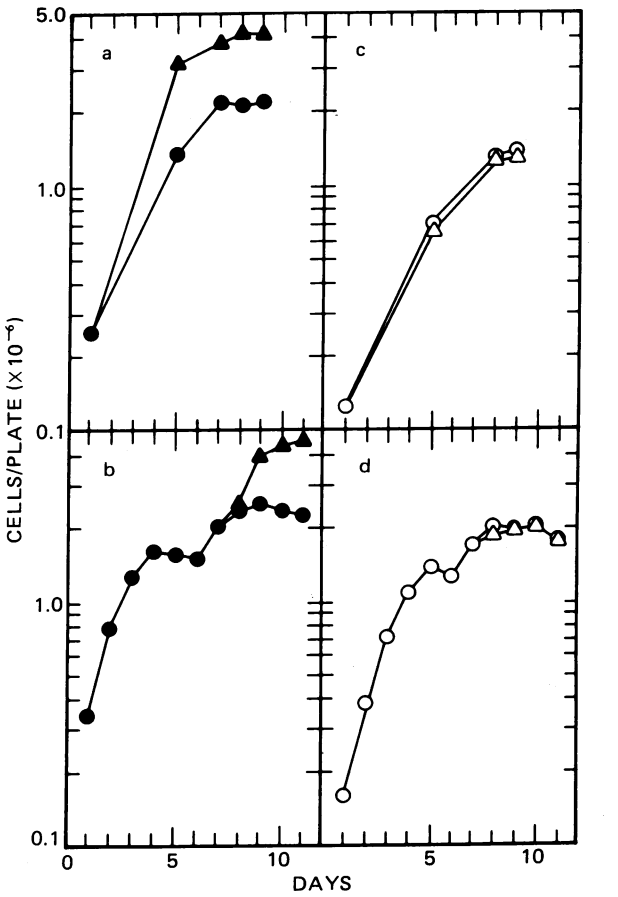

FIG. 2. Growth curves with and without EGF (10 ng/ml) for 3T3 and a nonproliferative variant, NR-6. (*a* and *c*) Cells were grown continuously with EGF; (*b* and *d*) EGF was added to confluent cultures. ▲ and △, EGF-treated cells; ● and ○, control cells. Closed symbols (*a* and *b*) illustrate growth curves on 3T3 cells; open symbols (*c* and *d*) illustrate growth curves on NR-6. All cell counts represent the average of duplicate plates.

Mitogenic Response of EGF Nonresponder Variants. We first examined the ability of three other reported 3T3 mitogens, PMA (14), PGF2α (15), and insulin (16–19), to stimulate divi-

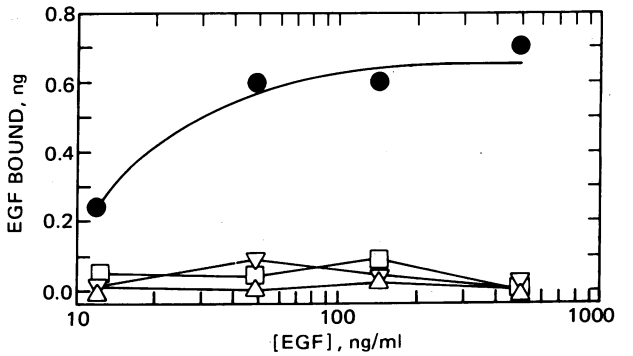

FIG. 3. Binding of ^{125}I-labeled EGF to confluent cultures of 3T3 (●) and to three nonresponder cell lines, NR-1 (△), NR-6 (▽), and NR-8 (□). The specific binding in the presence and absence of a 10-fold excess of unlabeled EGF was determined as described in the *text*.

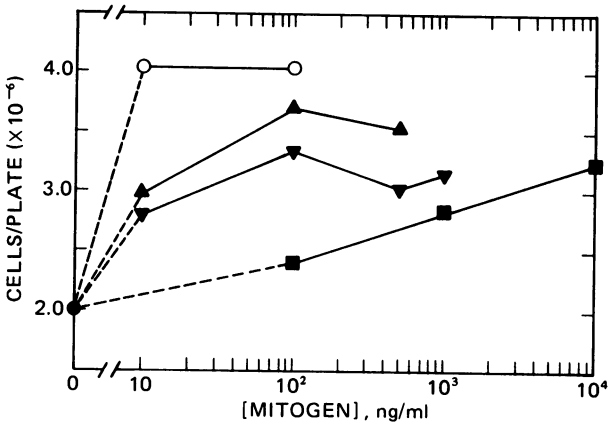

FIG. 4. The effect of different mitogens on the saturation density of 3T3 cells. Cells were grown in the presence of varying concentrations of three different chemically defined mitogens, PMA, PGF2α, and insulin as well as EGF. The mitogens were added to cells growing in 60-mm culture dishes in 5 ml of modified Eagle's medium containing 5% fetal calf serum. The final saturation density of the cells exposed to the various concentrations of PMA (▲), PGF2α (▼), and insulin (■) was compared to the saturation density of cells grown with 10 and 100 ng/ml of EGF (○) and to control cells (●).

sion of 3T3 cells (Fig. 4). The concentrations required for maximal stimulation of cell division are in the same range as reported by other workers. PMA and PGF2α were both maximally effective at a concentration of 100 ng/ml. Insulin showed the greatest effect on cell proliferation at 10 μg/ml. Using these concentrations, we then tested the eight nonresponder variant cell lines for mitogenic responses (Table 1). Although the eight nonresponders demonstrate no significant mitogenic response to EGF, they still retain ability to divide when stimulated by at least one of these other mitogens. Two nonresponder variant cell lines are capable of responding to all three of the other mitogens.

DISCUSSION

We have succeeded in selecting eight 3T3 variant cell lines that have lost the ability to respond to EGF stimulation of cell division. The EGF receptor appears to be absent or incapable of binding EGF in all eight lines.

Although the saturation density and mitogenic response data (Table 1) suggest that our eight variant cell lines are different from one another, we cannot rigorously conclude that each line is the result of an independent event. It is possible, because of the selection and enrichment procedure used prior to cloning, that a subset of the eight variants described here may contain multiple isolates of a single event. Should all independent EGF nonproliferative variants exhibit loss of a functional receptor, such an observation would have profound consequences for alternative models of mitogen modes of action.

We chose the colchicine selection technique in order to preserve a potential class of variants that might begin to synthesize DNA in response to EGF but is then blocked at a step between initiation of DNA synthesis and mitosis. The more commonly used selection techniques, which are designed to kill S-phase cells [reviewed by Thompson and Baker (20)], would eliminate such variants. A consequence of the colchicine selection technique apparently has been the selection of cell lines with lower saturation densities and reduced responses to increased serum. As cells divide in the presence of EGF and colchicine, they lose their ability to attach tightly to the surface of the dish. As a result, many of the cells float off the dish. Neighboring cells that were not stimulated to divide by EGF may initiate cell division as a result of the increased surface area, in a manner similar to the proliferation that occurs after the "wounding" of quiescent confluent cell layers (21–24). The colchicine technique may select for cells that do not respond to EGF and for cells that become quiescent at relatively lower saturation densities.

Four of the nonresponders exhibit a significant response to PGF2α, a mitogen that binds to a specific receptor on the plasma membrane of corpus luteum cells (25–27). The functional receptors for EGF and PGF2α are, therefore, clearly different. Those EGF nonresponsive variants that respond to PGF2α clearly still retain the ability to transduce mitogen receptor binding into activation of the events necessary to initiate cell division. The selection of an apparent "double nonresponder" such as NR-2, which responds to neither EGf nor PGF2α, might be the consequence of a block in a common step required for the insertion of both EGF and PGF2α receptors into the plasma membrane. Alternatively, these two distinct receptor activities could reside on the same macromolecule.

The variant cells described here, which no longer demonstrate EGF receptors, have served as excellent specificity controls for studies of EGF-induced alterations in membranes (A. Aharonov, I. Vlodavsky, R. M. Pruss, C. F. Fox, and H. R. Herschman, unpublished) and identification of the EGF receptor (8). This procedure for the selection of nonproliferative variants should be generalizable to other mitogens (e.g., PGF2α, PMA, insulin, and fibroblast growth factor). One should be able to produce variant cell lines missing functional receptors for all mitogens with a receptor-mediated mode of action. Such variants will be valuable in characterizing the specificity of presumptive mitogen-initiated events.

Modifications of this procedure should permit selection of

Table 1. Response of 3T3 and the nonproliferative variants to other mitogens*

Cell line	5% serum	10% serum	EGF	Insulin	PGF2α	PMA
3T3	1.75	3.36(92)	3.75(114)	2.90(66)	4.65(166)	3.55(103)
NR-1	1.33	2.08(56)	1.40(5)	1.71(29)	1.75(32)	2.33(75)
NR-2	1.13	1.67(48)	1.12(0)	1.19(5)	1.18(4)	1.76(56)
NR-3	1.10	1.71(55)	1.26(15)	1.39(26)	4.80(336)	2.05(86)
NR-4	1.30	2.19(68)	1.28(0)	1.43(10)	2.79(115)	2.79(115)
NR-5	1.15	1.88(63)	1.13(0)	1.55(35)	3.43(198)	2.99(160)
NR-6	1.43	2.42(69)	1.41(0)	1.61(13)	4.75(232)	2.50(75)
NR-7	1.60	2.25(41)	1.55(0)	1.95(22)	1.94(21)	2.19(37)
NR-8	1.35	2.45(81)	1.50(11)	1.75(30)	1.80(33)	2.56(90)

* Data are given as cells × 10^{-6} at confluence per 60-mm dish. Numbers in parentheses are the percent increase in cell number relative to the 5% serum control for each line.

sequentially isolated and stimultaneously selected "double nonresponders". Appropriate adaptation of the technique should also permit selection of conditional variants in a variety of steps resulting from the initiation of proliferation by specific mitogens. The phenotypic characterization of such variants will be of great value in our understanding of the mode(s) of action of mitogens.

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