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EFFECT OF 3T3 PLASMA MEMBRANES ON CELLS EXPOSED TO EPIDERMAL GROWTH FACTOR

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SUMMARY

Epidermal growth factor (EGF) induced DNA synthesis in non-confluent, G_0-arrested Swiss 3T3 fibroblasts is partially blocked by plasma membranes isolated from the EGF receptor deficient NR-6 Swiss 3T3 cell line. This inhibition could be due to either a steric block of the receptor by the membranes, a membrane induced down regulation of the EGF receptor, or a signal generated by membrane binding which is antagonistic towards the mitogenic signal generated by EGF. Binding measurements utilizing ^{125}I-labeled EGF demonstrated that membranes do not block either the EGF induced down regulation of the receptor or alter the number of receptors on the surface. These results suggest that the membranes exert their inhibitory effect via generation of a signal which is antagonistic to the EGF induced mitogenic signal, with the result expressed as a reduced mitogenic response.

Mouse epidermal growth factor (EGF)[1] has been shown by a number of investigators (1-3) to be a potent mitogen for Swiss 3T3 cells. We have recently demonstrated that the addition of a plasma membrane enriched fraction from Swiss 3T3 cells to sparse 3T3 cells results in the inhibition of DNA synthesis in these cells (4,5). Evidence has been presented that this inhibition of DNA synthesis parallels that observed when cells reach confluency, suggesting that membrane contact events play an important role in growth control (5). We have also demonstrated that the addition of serum or the combination of fibroblast growth factor (FGF) and dexamethasone can partially reverse the membrane effect (5).

One of the mechanisms by which membranes could block the effect of mitogens on cells is by preventing access of the mitogen to the cell surface.

[1]Abbreviations: EGF, epidermal growth factor; FGF, fibroblast growth factor; BSA, bovine serum albumin; DME, Dulbecco's modified Eagle's medium. PDE, phosphodiesterase units. One unit catalyzes the hydrolysis of 10 nmol of thymidine 5'-monophospho-p-nitrophenol ester per hour at 37°.

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The existence of a mutant of 3T3 cells, NR-6, which lacks the EGF receptor (6), allows experiments to be carried out with membranes which by themselves cannot bind EGF. Previous experiments by Fox et al. (7,8), in which confluent serum starved cultures were exposed to NR-6 membranes for 3 hours, showed that under those conditions membranes appeared to down regulate the EGF receptor. If this mechanism were generally applicable, then membrane inhibition of cell growth could be explained by a decrease in the concentration of mitogen receptors. We have examined the effect of membranes on EGF binding and present evidence in this communication that, under our assay conditions using sparse cells, membranes, which prevent the mitogenic effect of EGF, do not block binding of EGF to cells, nor do they reduce the number of EGF receptors present on the cell surface. In addition, membranes do not prevent down regulation by added EGF.

MATERIALS AND METHODS

Cells and Cell Culture: Swiss 3T3 cells were grown and kept in culture as previously described (4). The NR-6 derivative of Swiss 3T3 cells, which was obtained from Dr. C.F. Fox (UCLA), was cultured in the same manner as stock 3T3 cells. The NR-6 line does not contain demonstrable cell-surface receptors for EGF (6).

Preparation of Plasma Membranes and Addition of Membranes to Cells: Fractions containing enriched plasma membranes were prepared from confluent monolayers of NR-6 cells (30 150 mm diameter Falcon dishes) as previously described (9). As the membranes were prepared in the presence of bovine serum albumin (BSA) the quantity of membranes is determined by phosphodiesterase units, a plasma membrane marker. Only the fraction designated as Band I was used in these experiments. For addition to cells, membranes were washed once in DME/5% calf serum or DME/1% calf plasma and diluted to various concentrations in the appropriate medium in the presence or absence of EGF. Membranes were then sterilized by UV-irradiation and added to either 35 mm dishes (1.2 ml, for ^{125}I-EGF binding studies), or 1.7 cm Linbro wells (0.3 ml, for DNA synthesis measurements). Control dishes received fresh media (without membranes) treated as described above.

EGF and ^{125}I-labeled EGF: EGF was purified from male mouse submaxillary glands (Pel-Freeze) as described by Savage and Cohen (10). ^{125}I-EGF was prepared through the use of enzymo-beads (Bio-Rad), an immobilized lactoperoxidase and glucose oxidase catalyzed iodination system. The iodination of EGF was done according to the manufacturer's supplied recommendation. Briefly, 25 µl of reconstituted enzymo-beads were added to a solution containing 2.5 mCi ^{125}I (New England Nuclear), 10 µg EGF, 68 mM potassium phosphate buffer, pH 7.4, and 0.17% B-D(+)-glucose in a final volume of 145 µl. After a 30 minute incubation at room temperature, the beads were centrifuged (150 x g, 5 minutes) and the supernatant applied to a 0.8 x 14 cm Sephadex G-10 column equilibrated in 50 mM potassium phosphate, 0.1% BSA, pH 7.4. The labeled EGF

was eluted with the same buffer. Fractions containing EGF were pooled and stored at -20° for not longer than 2 weeks. These preparations had a specific activity of 30 to 60 μCi/μg EGF, and 80 to 90% of the counts were precipitable by trichloroacetic acid. Labeled EGF prepared in this manner had the same mitogenic activity towards 3T3 cells as native EGF (data not shown).

^{125}I-EGF Binding Assays: The growth medium was removed from the dish and the cells were washed one time with binding medium (DME containing 0.1% globulin-free BSA and 12.5 mM HEPES, pH 7.4). For measurement of total binding, 1.0 ml of binding medium containing ^{125}I-EGF (60 ng/ml) was placed over the cells and the dish incubated at 37° for 30 minutes. Unbound radioactivity was then removed by five 1.0 ml washes with ice-cold binding medium and the cells solubilized in 1.0 ml 2% Na_2CO_3/0.1 N NaOH for 30 minutes at 37°. The radioactivity of the entire amount was determined in a γ-counter.

Non-specific binding was determined by measuring the amount of ^{125}I-EGF bound in the presence of 5 μg/ml unlabeled EGF at 37°. Prior to the addition of the ^{125}I-EGF the dishes were pre-incubated for 2 minutes at room temperature with 5 μg/ml unlabeled EGF, and then the solution replaced with one containing 5 μg/ml unlabeled EGF and 60 ng/ml ^{125}I-labeled EGF. The amount of non-specific binding was subtracted from the total binding to give specific binding. For these experiments on sparse cultures, the non-specific binding varied between 10 and 25% of the total binding.

When binding was assayed on samples previously exposed to unlabeled EGF (i.e. down regulation), the dishes were washed 3 times, at 30 minute intervals, with fresh growth medium at 37° before the binding assay was started in order to remove the free hormone and to allow dissociation of the bound material.

Assay for DNA Synthesis: DNA synthesis in growing cells was assayed as previously described (4). To measure the stimulation of DNA synthesis by EGF cells were arrested early in the G_1 phase of growth by washing the dishes two times with DME to remove residual serum and then adding medium containing 1% calf plasma. The calf plasma (Gibco) was processed as described previously (5) except that the material was also passed over a CM-Sephadex column (500 ml capacity for 200 mls plasma) as outlined by Ross et al. for human plasma (9). After the cells had been exposed to 1% calf plasma for 48 hours, the medium was changed to 1% calf plasma containing EGF (either 10 or 60 ng/ml) in the presence or absence of exogenous membranes. DNA synthesis was then measured during a 4-hour pulse with 3 μCi/ml [^{3}H]-thymidine (5 Ci/mmole, Amersham), 19 to 23 hours after EGF addition. All DNA measurements were carried out with cells labeled to constant specific activity with [^{35}S]-methionine (7). Thus, the ratio of [^{3}H]- to [^{35}S]- in trichloroacetic acid insoluble material represents thymidine incorporation into DNA per unit protein.

RESULTS AND DISCUSSION

Addition of a plasma membrane enriched fraction prepared from NR-6 cells to growing 3T3 cells results in an inhibition of DNA synthesis, as previously observed with homologous membranes (Fig. 1A). The inhibition can be partially reversed by the addition of saturating concentrations of EGF, similar to previous observations with FGF and dexamethasone (5).

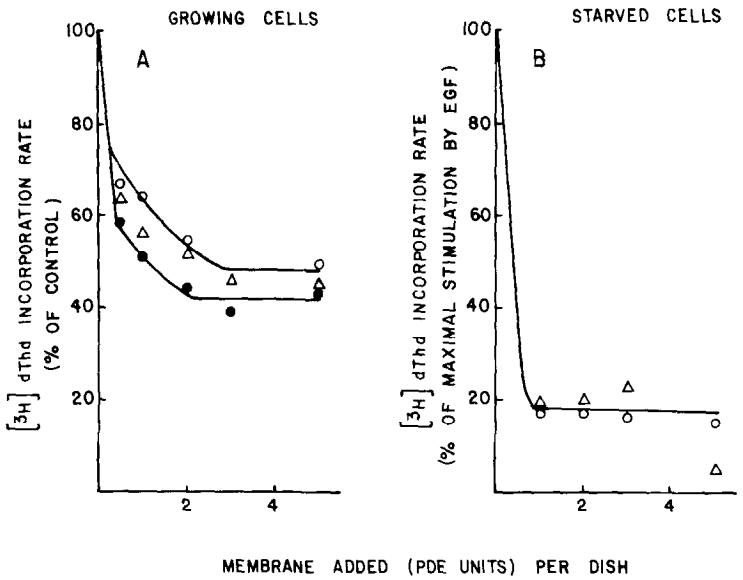

Fig. 1. Effect of NR-6 membranes on DNA synthesis. A) Effect of membrane addition to growing cells: 3T3 cells were initially plated in Linbro 24 well dishes at 1.0 x 10^3 cells per well. Twenty-four hours later, the medium was changed to 5% calf serum, and 24 hours after that membranes (± EGF) were added in 0.3 ml per well. The rate of DNA synthesis was determined 19 to 21 hours after membrane addition as described in the text. B) Effect of membranes on the mitogenic response of quiescent cells to EGF: 3T3 Cells were initially plated at 1.3 x 10^3 cells per well and 72 hours later the media was changed to DME-1% plasma derived serum. Forty-eight hours later, membranes ± EGF were added to the cells and the rate of DNA synthesis determined 19 to 23 hours after membrane addition. The stimulation by EGF was 3.3-fold as compared to cells not receiving EGF (determined in the absence of membranes). As the additions of high serum results in a 10-fold increase in DNA synthesis, we estimated that the addition of EGF is allowing approximately 30% of the cells to synthesize DNA during the 4-hour pulse. Symbols: ●, no EGF present; O, EGF present at 10 ng/ml; Δ, EGF present at 60 ng/ml.

To examine the question of whether membranes bring about the down regulation of the EGF receptor or restrict the access of EGF to the receptor, we have performed an analysis of the binding of ^{125}I-EGF to growing cells (Table 1), which demonstrated that the addition of membranes to cells did not down regulate the number of EGF receptors up to 19 hours after membrane addition, whereas the simultaneous addition of membranes and EGF led to a 75% reduction in binding sites within 5 hours. The slight decrease in the ^{125}I-EGF binding observed with the membrane treated sample 5 hours after membrane addition was not always observed in other experiments; the average (percent of control binding) value for this point (4 experiments) was 94 ± 7. These results sug-

TABLE 1

^{125}I-EGF Binding to Growing Cells

	Hours after addition of membranes	
	5	19
Sample	Specific ^{125}I-EGF bound, cpm/cell	
Control	0.29	0.22
+ Membranes (20 PDE units)	0.25	0.27
+ Membranes (20 PDE units) and EGF (60 ng/ml)	0.07	-

Specific binding was determined as described in the text. The specific activity of the ^{125}I-EGF was 52 μCi/μg. Cell number at 5 hours was 67,900 per dish for the control. At 19 hours the control contained 124,833 cells, the dishes receiving membranes 97,433. Cell counts were determined on triplicate samples using a Coulter Counter. Control cells received 1.2 ml fresh media when the membranes were added.

gest that membranes do not block EGF from gaining access to its receptor (otherwise down regulation by EGF would not have been observed) and that membranes do not alter the number of EGF receptors on the cell surface. In these experiments membranes and EGF were simultaneously added to the cells, and it is possible that EGF could bind to its receptor before maximum binding of membranes to the cells had occurred. We therefore repeated these experiments by preincubating cells for 24 hours with membrane, and then adding EGF (60 ng/ml) to the cells for 4 hours without changing the medium; therefore membranes were still present during the EGF incubation. Under these conditions EGF still down regulated its receptor. Cells in the absence of membranes were down regulated 78%; membrane treated cells 82%.

The preceding experiments were carried out in the presence of 5% calf serum, which contains many mitogenic factors and where it is not obvious that the membranes are only blocking the mitogenic response to EGF. The problem of multiple mitogenic species can be overcome by examining the effect of isolated plasma membranes on the EGF induced stimulation of DNA synthesis in cells starved for growth factors. Our starvation method involved placing the cells in calf plasma derived serum for 48 hours and then adding EGF (at either 10 or 60 ng/ml) and membranes to the cells. DNA synthesis was assayed 19-23

TABLE 2

^{125}I-EGF Binding to Quiescent Cells

	Hours after addition of membranes	
	5	19
Sample	Specific ^{125}I-EGF bound, cpm/cell	
Control	0.19	0.18
+ Membrane (20 PDE units)	0.15	0.21
+ Membranes (20 PDE units) and EGF (60 ng/ml)	0.05	-

Specific binding was determined as described in the text. The specific activity of the ^{125}I-EGF was 41 μCi/μg. Cell number at 5 hours was 69,800 for the control. At 19 hours the cell number was 68,600 for control and 71,700 for dishes which received membranes. Cell number determinations were done as described in the legend to Table 1. Quiescent cells were prepared as described in the text.

hours post EGF addition. Typical results are shown in Fig. 1B. Under these conditions the addition of membranes resulted in an 80% reduction of the EGF stimulation of DNA synthesis. There was no difference in inhibition when either 10 ng/ml of EGF was present (which is saturating for the mitogenic effect (2)) or if 60 ng/ml of EGF was present (which is saturating for binding (2)), implying that the membranes are interfering with the mitogenic signal EGF imparts to the cell. The data in Table 2 demonstrate that the reduction in stimulation of DNA synthesis is not due to down regulation of the EGF receptor by membranes, as the amount of ^{125}I-EGF bound after 19 hours is similar in both control and membrane treated cultures. Although a slight decrease in the level of the EGF receptor was noted in the experiment in Table II, this was not a reproducible phenomenon and the average value for 3 experiments, expressed as percent of control binding at 5 hours post membrane addition, was 93 $\pm$ 15.

We have also measured the level of the EGF receptor in 3T3 cells 1 hour after addition of membranes, and again find no change in the apparent number of EGF receptors under these conditions (data not shown). Simultaneous addition of EGF and membranes to starved cells resulted in a loss of receptors (Table II), as observed previously with growing cells. Thus, under experimental

conditions where the entire mitogenic stimulus is due to EGF, the presence of membranes is capable of blocking the mitogenic effect approximately 80% without preventing the mitogen from reaching its receptor. These data suggest that the membranes act via a signal to the cell which is antagonistic to the mitogenic signal of EGF, with the outcome expressed as a reduced mitogenic response.

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REFERENCES

1. Rose, S.P., Pruss, R.M., and Herschman, H.R. (1975) J. Cell. Physiol. 86, 593-598.
2. Aharonov, A., Pruss, R.M., and Herschman, H.R. (1978) J. Biol. Chem. 253, 3970-3977.
3. Carpenter, G., and Cohen, S. (1979) Ann. Rev. Biochem. 48, 193-216.
4. Whittenberger, B., and Glaser, L. (1977) Proc. Natl. Acad. Sci. USA 74, 2251-2255.
5. Whittenberger, B., Raben, D., and Glaser, L. (1979) J. Supramol. Struct. 10, 307-327.
6. Pruss, R.M., and Herschman, H.R. (1977) Proc. Natl. Acad. Sci. USA 74, 3918-3921.
7. Peterson, S.W., Vale, R., Das, M., and Fox, C.F. (1978) J. Supramol. Struct. (Suppl. 2), 126.
8. Fox, C.F., Vale, R., Peterson, S.W., and Das, M. (1979) Cold Spring Harbor Symp. Quant. Biol., in press.
9. Lieberman, M.A., Raben, D.M., Whittenberger, B., and Glaser, L. (1979) J. Biol. Chem. 254, 6357-6361.
10. Savage, C.R., Jr., and Cohen, S. (1972) J. Biol. Chem. 247, 7609-7611.
11. Ross, R., Nist, C., Kariya, B., Rivest, M.J., Raines, E., and Callis, J. (1978) J. Cell. Physiol. 97, 497-508.