

## INCREASED EPIDERMAL GROWTH FACTOR BINDING TO G<sub>0</sub>-ARRESTED 3T3 CELLS

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### 1. Introduction

Epidermal growth factor (EGF) is a potent mitogen for a variety of cell types [1] including Swiss 3T3 [2] and Balb/c 3T3 [3–5] fibroblast cell lines. Recent experiments [3–5] have shown that confluent Balb/c 3T3 cells, which are arrested early in the G<sub>1</sub> portion of the cell cycle (G<sub>0</sub>), have an increased number of EGF receptors relative to sparse growing cells. Addition of a plasma membrane-enriched fraction derived from confluent Swiss 3T3 cells to sparse-growing 3T3 cells arrests 50% of the cell population in G<sub>0</sub> during each cell cycle [6]. We show here that sparse cells arrested in G<sub>0</sub> by the addition of plasma membranes, or by culture in the absence of platelet-derived growth factor (PDGF), exhibit an increased binding of EGF relative to sparse, growing cells. We conclude that arrest in G<sub>0</sub> results in an up-regulation of the EGF receptor in Swiss 3T3 cells and that this effect is independent of the agent used to arrest cells in G<sub>0</sub>.

### 2. Materials and methods

Published procedures were used for the growth of 3T3 cells and for the preparation of plasma membrane-enriched fractions from these cells [7,8]. NR-6 cells are a variant of 3T3 cells which lack the receptor for EGF [9]. The binding of <sup>125</sup>I-labeled EGF to cells was done as in [8]; the level of non-specific binding was determined by the addition of non-radioactive EGF at 5 µg/nl. The level of non-specific binding is approximately the same per tissue culture dish for both sparse and confluent cultures, therefore the non-specific binding represents a greater fraction of the total binding for sparse cultures than for confluent

cultures. All binding experiments were done in triplicate, and the correction for non-specific binding is indicated in each case.

Purified platelet derived growth factor (PDGF) [10] was prepared by an unpublished procedure and was purified ~20 000-fold from a crude platelet extract (T. D. et al., in preparation).

### 3. Results and discussion

Upon EGF binding to the appropriate cell surface receptor [11–14] the EGF receptors rapidly patch [7,8], become internalized via coated pits [9–13], and are degraded within the lysosomes [13–15]. It is not yet clear whether any steps of the above pathway, other than binding, is required for EGF to elicit a mitogenic response [16–21]. The internalization process does, however, lead to a reduction of EGF receptors on the cell surface, a process which had been termed down-regulation [1,2]. The receptors for various peptide hormones [22,23], particularly fibroblast growth factor (FGF) [24], platelet-derived growth factor [10], and EGF, are co-regulated; i.e., addition of one hormone can either down-regulate or up-regulate the receptors for the other two hormones. Thus, the regulation of the EGF receptors on the cell surface appears to be complex. To determine what effect the position in the cell cycle has on the binding of EGF to the cell surface we measured the binding of EGF to 3T3 cells in 4 stages of growth; exponential, density-arrested cells, membrane-arrested sparse cells, and mitogen-depleted (which leads to G<sub>0</sub> arrest) sparse cells. Addition of membranes to cells results in arrest of cells in G<sub>0</sub> without restricting the access of EGF to the cell surface [8]. The data are represented in tables 1–3.

The binding of EGF to cells in tissue culture is rapidly followed by internalization of the bound EGF. In Balb/c 3T3 cells 50% of the cell-associated hormone was shown to be internal after 10 min incubation at 23°C [25]. Using the same method we have found for confluent Swiss 3T3 cells that 90% of the cell-associated EGF is internal after 10 min incubation at 37°C; by contrast, <10% of the cell-associated hormone is internal after 60 min incubation at 4°C. Thus, we carried out binding assays both at 37°C and 4°C.

Addition of membranes to 3T3 cells arrests growth by a mechanism which resembles that observed at high cell density [6,7]. The data in tables 1 and 2 show that either at high cell density or after addition of membranes, the apparent number of EGF receptors is increased relative to sparse growing cells. The data in table 1 were obtained at 4°C and are a measure of the number of receptors exposed on the cell surface, while the data at 37°C represent a combination of cell surface receptors and internalized hormone. The membrane preparation used in these experiments was derived from NR-6 cells, a variant obtained from 3T3 cells which lacks the EGF receptor [9], thus cell-associated membranes do not con-

Table 1  
Binding of <sup>125</sup>I-labeled EGF to Swiss 3T3 cells at 4°C

Cells	<sup>125</sup> I-labeled EGF bound (molecules/cell)	(%)
Sparse, growing	34 400	100
Sparse, + membranes	68 370	198
Dense, arrested	68 840	197

Sparse cells were plated at  $2.4 \times 10^3$  cells/35 mm dish (2 ml/dish); dense cells at  $8 \times 10^4$  cells/35 mm dish in Dulbecco's modified Eagle's medium (DME) 10% calf serum. At 24 h after plating all dishes were fed with DME/5% calf serum. After an additional 24 h at 37°C membranes, from NR-6 cells, were added to some dishes (11.75 phosphodiesterase units/dish, 1.5 ml/dish, in DME/5% calf serum), while the other dishes received 1.5 ml DME/5% calf serum. At 48 h after membrane addition EGF binding was determined as described previously utilizing <sup>125</sup>I-labeled EGF at 25 ng/ml with spec. act. 96.8 µCi/µg. The binding was at 4°C for 60 min. The non-specific binding was 50% for sparse cultures, 10% for dense cultures. Cell numbers were 53 700 for control cells, 30 150 for membrane-treated cells, and 330 000 for dense cells. Rates of [<sup>3</sup>H]thymidine incorporation into DNA were also determined [8] and they were, relative to the sparse-growing control, 47% for membrane treated cells and 15% for the dense cells. This experiment was repeated 3 times with similar results

Table 2  
Binding of <sup>125</sup>I-labeled EGF to Swiss 3T3 cells at 37°C

Cells	<sup>125</sup> I-labeled EGF bound (molecules/cell)	(%)
Sparse	148 400	100
Sparse, + membranes	233 800	158
Dense, arrested	312 100	210

Sparse cells were plated at  $4 \times 10^3$  cells/35 mm dish (2 ml/dish) and dense cells at  $6 \times 10^4$  cells/dish in DME/5% calf serum. After 48 h NR-6 membranes were added to some dishes at 20 PDE/dish, 1.2 ml/dish. All other dishes were fed with DME/5% calf serum, 1.2 ml/dish. Two days after membrane addition EGF binding was determined using <sup>125</sup>I-labeled EGF at 40 ng/ml, 56.1 µCi/µg. Non-specific binding was 40% for the sparse cultures, 7% for the dense. Cell numbers at the time of assay were 36 400 for control, 23 000 for membrane-treated and 190 000 for dense cultures. Membrane treated cultures had a DNA synthesis rate 30% that of control; dense cells were at 20% of control. This experiment was repeated 6 times with essentially similar results

tribute to the observed binding of EGF. We have considered the possibility that the difference in surface exposure of the EGF receptor in confluent and sparse cells is artifactual and could be due to carry-over into the assay of serum components which have bound to the EGF receptor, and that a higher concentration of these components are present over sparse growing cells as compared to confluent cells. We have measured binding of EGF to sparse and confluent cells in the presence of 1% serum, and find at most a 10% reduction in binding for both sparse and confluent cells (not shown).

The data in tables 1–3 have been obtained at saturating or nearly saturating levels of EGF. If the change in EGF binding reflects a change in the affinity of the receptors for EGF, then the difference between growing and arrested cells should be accentuated at low [EGF]. Accordingly, we have also carried out binding experiments at 5 ng EGF/ml and have observed the same differences in binding between arrested and growing cells as at higher [EGF] (table 4). Thus the effect of arresting cells at  $G_0$  is primarily on the number of binding sites and not their affinity for EGF.

Arrest of cells by high cell density is accompanied by a number of physiological changes of which alterations in the rate of uptake for solutes such as amino acids or glucose are an example [26]. Such changes could be due to either a density effect per se or a consequence of cell arrest at  $G_0$ . Cell density affects

Table 3  
Binding of  $^{125}\text{I}$ -labeled EGF to arrested and stimulated  
3T3 cells at  $4^\circ\text{C}$

Cells	$^{125}\text{I}$ -labeled EGF bound		DNA synthesis rate (%)
	(molecules/cell)	(%)	
Sparse, growing	38 600	100	100
Dense, arrested	109 800	284	14
Sparse, starved	173 500	449	18
+ PDGF (4 h)	142 800	370	12
+ PDGF (16 h)	183 720	476	85

Sparse cells were plated at  $2.4 \times 10^3$  cells/35 mm dish; cells to be starved were plated at  $4.8 \times 10^3$  cells/35 mm dish, and dense cells were plated at  $8 \times 10^4$  cells/dish (at 2 ml/dish), all in DME/10% calf serum. Two days after plating some cells were starved by incubation in medium containing 1% plasma rather than serum and therefore lacking PDGF [10]. For this purpose cultures were washed twice with DME followed by 2.0 ml DME/1% calf plasma, prepared as in [8]. Control cultures received 1.5 ml fresh DME/5% calf serum. This concentration of serum gives a maximal mitogenic response for sparse cells. After 48 h EGF binding and rates of DNA synthesis were determined. The  $^{125}\text{I}$ -labeled EGF was used at 50 ng/ml, 96.8  $\mu\text{Ci}/\mu\text{g}$  and binding done at  $4^\circ\text{C}$  for 60 min. Partially purified platelet factor (20 ng/ml) (PDGF) was added to some starved cells and the binding determined both 4 h and 16 h after PDGF addition. Non-specific binding was between 50–60% for sparse cells, 10% for dense cells. Cell numbers were 34 000 for control cells, 17 000–19 000 for starved cells and 300 000 for dense cultures. When binding was measured for 30 min at  $37^\circ\text{C}$  control cells bound 0.17 cpm/cell (100%), whereas starved cells bound 0.61 cpm/cell (360%). These experiments have been done twice with identical results

Table 4  
Binding of  $^{125}\text{I}$ -labeled EGF to 3T3 cells at 5 ng EGF/ml and  
50 ng EGF/ml

Cells	$^{125}\text{I}$ -labeled EGF bound	
	5 ng/ml (molecules/cell)	50 ng/ml (molecules/cell)
Sparse, growing	18 600	38 600
Sparse + membranes	37 200	99 000
Sparse, starved	55 800	173 000
Dense, arrested	37 200	109 000

The protocol for this experiment was as in table 1. Binding of  $^{125}\text{I}$ -labeled EGF was measured at  $4^\circ\text{C}$  for 60 min. The level of non-specific binding at 5 ng EGF/ml was 25% for sparse cells and confluent cells

the rate of  $\alpha$ -aminoisobutyric acid and glucose transport in cells already arrested in  $G_0$  [27]. To answer this question (cell density vs  $G_0$  arrest) regarding the increase in the level of EGF receptors at confluency, we have measured the level of EGF receptor in sparse cells which were arrested in  $G_0$  by incubation in 1% calf plasma, which lacks the major mitogen for 3T3 cells present in serum (platelet derived growth factor (PDGF)) [10]. Representative data are shown in table 3. Arrest of cells in  $G_0$  by removal of PDGF also results in an increased level of EGF binding to the cells. Thus we conclude that arrest of cells in  $G_0$ , whether by density or by limiting concentration of a mitogen, is sufficient to increase the level of the EGF receptor. Addition of PDGF to plasma-arrested cells results in a synchronous re-entry of the cells into the cell cycle. The data in table 3 show that either 4 h after PDGF addition or 16 h after PDGF addition the number of cell surface receptors for EGF remains high. At 4 h after EGF addition the cells are still in  $G_1$  and at 16 h are mostly in the S phase of the cell cycle scheme. At this time one observes the highest rate of DNA synthesis after addition of PDGF to the cells. Thus, down-regulation of the EGF receptor to a level comparable to that observed in sparse-growing cells is not an immediate consequence of the initiation of growth following mitogen stimulation nor does it reflect an obligatory event at the cell surface required for the initiation of cell growth.

Increased levels of mitogen receptor at the cell surface under conditions of growth arrest can be considered to represent a compensatory mechanism such that at a given concentration of hormone or mitogenic factor more sites per cell would be occupied; thus, even at sub-optimal hormone levels sufficient sites may be occupied to elicit a mitogenic response. Hormone or mitogen receptors in a number of instances have been shown to be down-regulated as a consequence of occupancy of the receptor [1,28]. These observations suggest that the level of the EGF receptor can also be controlled by other ligands that do not bind to the EGF receptor, including surface components from other cells (contact inhibition of growth) and mitogenic components present in serum. Relevant to this argument is the demonstration that binding of NR-6 membranes to 3T3 cells does not prevent access of EGF to the cell surface [8]. The opposite effect has been shown for BSC-1 cells [29]; as these cells reach confluence the EGF receptors on the cell surface decrease 10-fold. We interpret these

data to indicate that cell-cell contact leads to a down-regulation of EGF receptors in these cells; once the receptors are down-regulated the cells are less responsive to serum factors and undergo growth arrest. The opposite, however, appears to be the case for 3T3 cells; growth arrest by cell-cell contact leads to a compensatory mechanism of receptor up-regulation, which could lead to initiation of growth in response to sub-optimal levels of mitogens.

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