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Modulation of EGF Binding and Action by Succinylated Concanavalin A in Fibroblast Cell Cultures

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The role of the binding of succinylated concanavalin A to tissue culture cells in influencing epidermal growth factor (EGF)-mediated cell proliferation has been studied. Succinylated concanavalin A dramatically reduces the stimulation of 3T6 cells by EGF in Dulbecco's modified Eagle's medium (DME) containing insulin and vitamin B_{12} as additional growth factors, but no serum. Furthermore, binding studies using ^{125}I-labeled EGF have shown that the binding of EGF to the cell surface is reduced upon addition of succinylated concanavalin A.

Key words: growth regulation, epidermal growth factor, density inhibition of growth, extracellular matrix

Many of the approaches to elucidate growth control in tissue culture in the last few years can be grouped into two categories. The first one considers the availability and binding of various nutrient and/or regulatory factors to the cell surface or the cell in general. The second one is concerned with the role of cell–cell interactions in the regulation of cell growth. The two concepts do not necessarily have to be mutually exclusive. Modifications or perturbations of the cell surface which lead to changes in the growth control of the cells might give some clues as to how membranes can regulate cell growth.

It has been shown earlier that a nontoxic lectin-derivative of concanavalin A (succinyl-Con A) is able to inhibit cell growth reversibly in a density-dependent manner in various cell lines [1–3]. Since epidermal growth factor (EGF) acts by first binding to a specific receptor on the cell surface, we decided to investigate the interaction of succinyl-Con A with binding and growth promotion by EGF. For these studies 3T6 cells were used since they can be stimulated to reassume DNA synthesis after serum starvation by the addition of pure mitogenic factors.

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MATERIAL AND METHODS

Cell Cultures and Materials

3T6 cells were cultured in Falcon tissue culture plates in Dulbecco's modified Eagle's Medium (Gibco) supplemented with 10% calf serum (Gibco). 3T6 cells were a gift from Dr. Rudland at the Imperial Cancer Research Fund in London. Cultures were unfrozen from stocks every 2–3 months and routinely checked for PPLO. Radioactive isotopes were from Amersham or Institut für Reaktorforschung, Würenlingen, Switzerland.

Con A and succinylated Con A were isolated and prepared as described previously [1]. Insulin, bovine serum albumin (BSA), and vitamin B_{12} were obtained from Sigma. Penicillin-streptomycin (1,000 units/ml) was from Gibco. EGF was isolated [4] and labeled [5].

Stimulation of Cells in Presence of Growth Factors

The 3T6 cells (2×10^5) were seeded in 2 ml DME plus 0.5% serum into 35 mm plates. After 3 days the cells were washed twice with serum-free medium, and 2 ml of DME plus 1% penicillin-streptomycin were added. EGF, insulin, and vitamin B_{12} were added 2 days later. The factors were added in phosphate-buffered saline (PBS) containing 0.1% BSA. At the same time the cultures obtained 2 μCi ^{3}H-thymidine (20 Ci/mmole). Succinyl-Con A was added to a final concentration of 200 μg/ml a few hours prior to factor addition.

The cultures were incubated for up to 48 h, fixed in PBS plus 3.5% formaldehyde, and prepared for autoradiography. After 10 days they were developed and stained with Giemsa. Five hundred to 1,000 cells were counted in duplicate samples; the standard deviation was always less than 10%. In 4 different experiments similar results were obtained.

Binding of ^{125}I-EGF

The 3T6 cells (2.5×10^5) were plated in 5 ml DME plus 10% serum into 6 cm plates. Three days later the medium was removed and changed to 2 ml serum-free DME. Succinyl-Con A was added (200 μg/ml) and ^{125}I-labeled EGF (specific activity 2×10^5 cpm/ng) was added in PBS containing 0.1% BSA 2–4 hours later. The total volume in the binding assays was 1 ml. Maximal binding occurred after 30–60 min at 37°C. The cells were washed 4 times with 5 ml ice-cold PBS and dissolved in 0.5 N NaOH. They were counted in a Packard Model 5385 γ-counter. Each point was determined in triplicate; the standard deviation was always below 10%. Similar results were obtained if the cells were kept in serum-free DME for 1 day prior to the addition of EGF.

RESULTS

In order to investigate the question whether succinyl-Con A affects cell stimulation in a defined tissue culture system, we used 3T6 cells, which can be arrested in G_0/G_1 by withdrawing serum for several days. Upon addition of EGF, insulin, and vitamin B_{12} they reenter the cell cycle within about 12 h. Insulin and vitamin B_{12} alone show no stimulatory activity if added in the absence of EGF. Without insulin and vitamin B_{12}, no growth stimulation could be observed after addition of EGF. Figure 1 shows the increase in the percentage of cells showing DNA synthesis after addition of different concentrations of EGF. Insulin and vitamin B_{12} were always added at concentrations of 100 ng/ml and 400 ng/ml, respectively. Above 2–5 ng/ml EGF the cells are not stimulated any further. Maximal stimulation using 3T6 cells achieves in the order of 80% labeled cell nuclei and occurs within 30 h. Longer incubation does not lead to a higher proportion of cells synthesizing DNA.

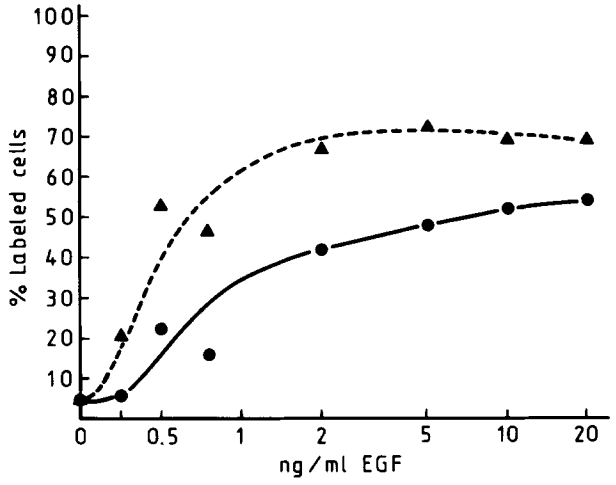

Fig. 1. 3T6 cells were plated and EGF, insulin, and vitamin B_{12} added as described in Material and Methods; (▲- - -▲) shows stimulation of cells after 30 h in the absence of succinyl-Con A, (●—●) shows cell stimulation in presence of 200 μg/ml succinyl-Con A. If no EGF was added, the labeling index was 4–5%, independent of whether insulin and vitamin B_{12} were present. This background stimulation was also independent of whether succinyl-Con A was present.

If resting 3T6 cells are treated with 200 μg/ml succinyl-Con A 2–4 h prior to addition of growth factors, they re-enter the cell cycle to a considerably lower extent. Figure 1 shows that the highest inhibition occurs between 0.2 and 2 ng/ml EGF added to resting cells. This is the concentration range shown to be below saturation of the biological effect of EGF on resting cells in the absence of succinyl-Con A. The maximal inhibition of EGF stimulation by succinyl-Con A is about 70% (at 0.25 ng/ml EGF). At very high EGF concentrations (above 20 ng/ml) the succinyl-Con A inhibition can be overcome, as is expected for inhibition of the competitive type.

When α-methyl-D-mannoside is added together with succinyl-Con A to the cells, it inhibits the effect of the lectin-derivative completely. The hapten sugar also abolishes the effect of succinyl-Con A if added after the cells are cultured in the presence of succinyl-Con A for several days [1–3]. We conclude therefore that succinyl-Con A does not inactivate EGF in the medium.

In order to investigate the interaction between EGF and the cell membrane, in the presence and absence of succinyl-Con A, we studied the binding of this growth factor to 3T6 cells. Succinyl-Con A clearly reduces the binding of ^{125}I-labeled EGF to its receptors. Figure 2 shows a plot of a representative binding experiment done with 3T6 cells. When 200 μg/ml succinyl-Con A was added to the cells 2–4 hours prior to EGF, it clearly reduced the amount of EGF bound to the cells. Succinyl-Con A has to be present in the binding medium during the whole incubation period with EGF. When the cells were treated with succinyl-Con A for longer periods prior to EGF addition no further inhibition of binding occurred. When α-methyl-D-mannoside was added to the binding medium together with succinyl-Con A, it prevented the lectin-induced decrease in EGF binding (data not shown). The binding data represented in Figure 2 show that the binding of EGR to 3T6 cells in the presence of succinyl-Con A is not saturated at concentrations up to 10 ng/ml. This may in

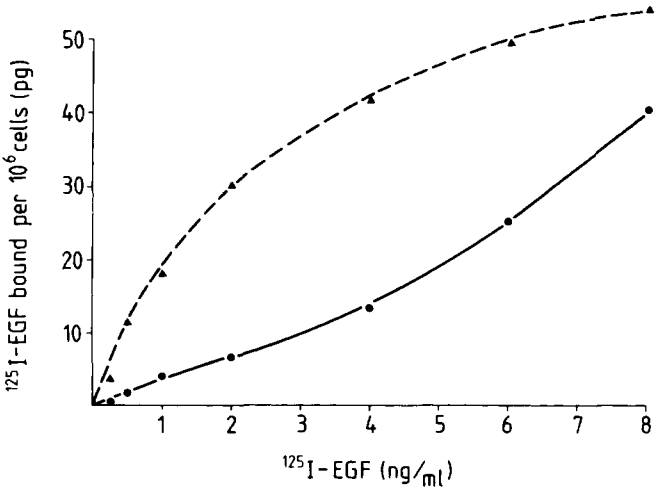

Fig. 2. Binding curve of ^{125}I-labeled EGF to 3T6 cells. Only the specific binding – ie, the amount of EGF bound after subtracting the percentage of cell-associated material in presence of a 100–500-fold excess of unlabeled EGF – is plotted; (▲- - -▲) shows binding to control cells; (●—●) shows binding to cells pretreated with 200 μg/ml succinyl-Con A.

part be related to the finding that succinyl-Con A increases the unspecific binding of the hormone to 3T6 cells. In our experiments unspecific binding was the amount of ^{125}I-EGF associated with the cells in presence of a 100–500-fold excess of cold material. Up to 70% of the cell-associated material was unspecifically bound in presence of succinyl-Con A (at 8 ng/ml EGF), whereas in control experiments it was always below 10%.

DISCUSSION

Succinyl-Con A is known to reversibly inhibit cell growth in vitro in tissue culture media containing calf serum, a complex mixture of exogenous growth factors [1–3]. Under these conditions succinyl-Con A mimicks some aspects of density-dependent growth control in untransformed and virus-transformed cells. Addition of a Con A-specific hapten sugar leads to release of most of the bound lectin, followed by reinitiation of cell growth [3]. This suggests that succinyl-Con A acts at the cell periphery. Several other authors have reported that lectins interfere with the binding of hormones to membrane receptors [6–8].

We investigated the influence of succinyl-Con A on the binding of a specific growth factor to the cell surface. To circumvent the problems arising from the use of complete (and therefore undefined) sera in growing cells in culture, we used a more defined system containing only 3 factors that can stimulate resting cells to enter S-phase [9]. Although EGF, insulin, and vitamin B_{12} stimulate a high proportion of G_0/G_1 cells to enter S-phase without serum addition, we were not able to monitor a fast increase in the mitotic rate of our cultures. Neither is it possible to maintain these cells for more than 10 days under serum-free conditions without a reduction in cell viability. We assume therefore that other factors are necessary to induce the whole cell cycle. Nevertheless it was possible to study the effect of succinyl-Con A with binding and stimulation of DNA synthesis after addition

of EGF, insulin, and vitamin B_{12} to resting 3T6 cells. The dose-response curve for EGF is clearly shifted to higher EGF concentrations (Fig. 1). Figure 2 shows that succinyl-Con A also influences the binding of EGF to the cell membrane and/or the subsequent degradation or uptake of the hormone-receptor complex.

To clarify further the role of succinyl-Con A in reducing the EGF-induced cell stimulation, several aspects have to be considered: 1) succinyl-Con A may reduce the binding of EGF to the cell membrane by an allosteric hindrance of the receptor; 2) succinyl-Con A may down regulate the number of EGF receptors – eg, via co-internalization of EGF receptors into lectin-induced endocytotic vesicles; 3) succinyl-Con A may interfere with the degradation of the EGF–receptor complex. All three points need further investigation.

Other authors have described the effect of tumor promoters on EGF binding or action in different tissue culture systems. These data suggest that phorbol esters interact indirectly with EGF receptors and do not bind to the receptor itself. In our system there is good correlation between succinyl-Con A–induced inhibition of cell stimulation and EGF binding. However, the possibility that other mechanisms are involved in growth regulation have also to be considered. Therefore no causal relationship between inhibition of EGF binding and the observed decrease in cell stimulation can be evoked.

The number of binding sites for lectins at the cell surface is much higher than for hormones like EGF [14, 15]. It is possible therefore that several other growth factor binding sites and/or other functions of the cell membrane are impaired by succinyl-Con A. The reversible growth regulation in serum containing tissue cultures observed earlier could be the reflection of many different molecular changes induced by succinyl-Con A on the cell surface. The data presented here do not allow a definite interpretation of the earlier finding that succinyl-Con A–regulated cell growth in a density-dependent way [1]. Other workers found a density dependence of the effect of EGF on cell proliferation [12, 13]. However, nothing is known so far about the mechanism by which cell density affects hormone receptors.

Other effects of succinyl-Con A have been found; among them is a reduction of cell mobility in dense but not in sparse culture (manuscript in preparation). This could proceed through an increase in cell-to-cell adhesion mediated by succinyl-Con A.

Other authors have discussed a possible role of the extracellular matrix or, more generally, of the microenvironment of the cell in growth regulation [16–20]. Since the extracellular matrix is rich in carbohydrate to which lectins can bind, we speculate that a change in the extracellular matrix may be related to the growth inhibition induced by succinyl-Con A. A not yet determined decrease in the affinity of the EGF receptors upon succinyl-Con A treatment may be the reflection of a general change in the structure of the extracellular matrix. The alternative possibility is a direct interaction of succinyl-Con A with the receptor molecules. No data are available so far that make it possible to distinguish between these two hypotheses.

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