

Contact Inhibition of Growth of Human Diploid Fibroblasts by Immobilized Plasma Membrane Glycoproteins

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Abstract. The human embryonic fibroblasts used in this study show pronounced inhibition of growth when reaching a critical cell density. High cell density and growth inhibition has previously been mimicked by the addition of glutaraldehyde-fixed cells or of isolated plasma membranes to sparsely seeded proliferating fibroblasts (Wieser, R. J., R. Heck, and F. Oesch, 1985, *Exp. Cell Res.*, 158:493–499).

In this report, we describe the successful solubilization of the growth-inhibiting glycoproteins and their covalent coupling to silicabeads (10 μm), which had been derivatized with 3-isothiocyanatopropyltriethoxysilane. The beads, bearing the plasma membrane proteins, were added to sparsely seeded, actively proliferating fibroblasts, and growth was measured by the determination of cell number or of incorporation of [^3H]thymidine into DNA. The growth was inhibited in a concentration-dependent manner, whereby 50% inhibition was achieved with 0.3 μg of immobilized

protein added to 5×10^3 cells. Terminal galactose residues of plasma membrane glycoproteins with N-glycosidically bound carbohydrates were responsible for the inhibition of growth.

Dense cultures of human fibroblasts are characterized by an accelerated synthesis of procollagen type III. We have found that this cellular response can also be induced by the addition of immobilized plasma membrane glycoproteins to sparsely seeded cells. These observations support the conclusion that the addition of immobilized plasma membrane glycoproteins to sparsely seeded fibroblasts mimics the situation occurring at high cell density. These results show that cell-cell contacts via plasma membrane glycoproteins carrying terminal galactose residues are important for the regulation of the proliferation of cultured human fibroblasts and presumably of the accelerated synthesis of collagen type III.

THE growth of normal cells in vitro is under highly effective control leading to the cessation of growth, when a cell- and culture condition-specific cell density is reached. Two major hypotheses attempt to explain this regulation: (a) the inhibition of growth by density-dependent mechanisms due to medium depletion (6) or to the accumulation of inhibitory molecules in the medium (7), or, (b) inhibition of growth by specific cell-cell interactions (contact-dependent inhibition of growth) via plasma membrane glycoproteins (5, 12, 17, 25). Although the molecules, which are supposed to be involved in the contact-dependent inhibition of growth, have only been partially purified (13, 18), there exists strong evidence that the inhibition of growth, exerted by plasma membranes or partially purified membrane proteins, is of a specific nature. This is shown by the fact that, aside from the inhibition of growth, additional events characteristic for cultures of high cell density can be induced by the addition of isolated plasma membranes or of solubilized plasma membrane molecules to sparsely seeded cells (12, 18).

Due to their hydrophobic properties, plasma membrane proteins tend to aggregate in detergent-free solution; e.g., when tested on their influence on cellular reactions. This

may lead to endocytosis resulting in unwanted cellular responses, not induced by cell-cell contacts. On the other hand, aggregation may lead to an underestimation of the purification factor, as the degree of inhibition of growth strongly depends on the number of imitated cell-cell contacts. In addition, from a physiological point of view, the molecules under investigation should be immobilized, so that only contact-dependent cellular reactions are induced, thereby preventing artifacts which may occur if the freely diffusible molecules are taken up by the cells.

Here we describe a method for the imitation of cell-cell contact by the addition of immobilized plasma membrane glycoproteins to sparsely seeded fibroblasts and their influence on the growth and on the synthesis of collagen type III.

Materials and Methods

Materials

All chemicals and biochemicals were purchased from Sigma Chemie GmbH (Taufkirchen, FRG), if not stated otherwise. A research sample of 3-isothiocyanatopropyltriethoxysilane was kindly provided by E. Merck,

Darmstadt, FRG. Cell culture plastics were purchased from Nunc, Wiesbaden, FRG.

Cell Culture

Human embryonal lung fibroblasts were cultured as described elsewhere (26).

Isolation of Plasma Membranes

Plasma membranes were isolated as described (4) with slight modifications. Briefly, confluent monolayers of human fibroblasts ($1.5\text{--}2 \times 10^5$ cells/cm²) were rinsed twice with cold Ca, Mg-free phosphate buffered saline (PBS), and collected by centrifugation after trypsinization (0.015% in PBS/0.002% EDTA). The cell pellet was washed once by centrifugation in attachment buffer (Dulbecco's modified Eagle's medium [DME]/310 mM saccharose, 7:3; vol/vol)¹ and diluted to a final concentration of 1×10^7 cells/ml attachment buffer. To the cell suspension 1×10^5 beads (Cytodex 1, Pharmacia, Freiburg, FRG; pre-swollen in PBS)/ml attachment buffer were added dropwise and incubated at room temperature while shaking on a rocker platform. After 15 min, the vial containing the cell-bead suspension was filled with ice-cold PBS, and the beads with attached cells were allowed to settle. The supernatant containing the cells not attached to the beads was removed by aspiration and the pellet washed three times with PBS. The final pellet was resuspended in 10 mM Tris/HCl, pH 9.0 (T-buffer), pelleted by centrifugation, resuspended in T-buffer, and agitated on a Vortex mixer for 30 s in order to break the cells. After three washes with T-buffer, the suspension was sonicated (Branson Sonifier, standard microtip; settings: output 2, duty cycle 30% [Branson Sonic Power Co., Danbury, CT]) with 30 pulses. After three additional wash steps in T-buffer, plasma membrane proteins were solubilized as described below.

Solubilization of Plasma Membrane Proteins

Proteins from plasma membranes, adhering to the microcarrier beads used for their isolation, were solubilized in solubilization buffer (PBS, pH 8.0; 4 mM 3-[(3-cholamidopropyl)-dimethylammonio]l-propane-sulfonate [CHAPS] (22); 1 mM phenylmethylsulfonyl fluoride; 0.02% NaN₃) for 30 min at 4°C. After solubilization, the supernatant containing the solubilized plasma membrane molecules was removed and cleared by centrifugation (43,000 g, 70 min).

Immobilization of Solubilized Proteins to Porous Silica

The binding of proteins to silicabeads was performed as described (9) with some modifications. Briefly, silicabeads (Lichrospher, Si 500 Å, 10 µm, E. Merck) were activated under reduced pressure (0.1 Pa) for 12 h at 200°C. After cooling, dichloromethane (5 mg/g silicabeads), dry 2,6-dimethylpyridine (0.4 mmol/g silicabeads) and 3-isothiocyanatopropyltriethoxysilane (0.4 mmol/g silicabeads), prepared as described by Vogt (24), were mixed under vacuum. After refluxing the suspension under dry argon for 24 h, the silicabeads were washed with chloroform, methanol, and water. The dried, "activated" carrier was stored until use at 4°C. The ligand density for 3-isothiocyanatopropyltriethoxysilane was determined by elemental analysis and shown to be $3.5 \mu\text{mol}/\text{m}^2$ (9). Covalent binding of solubilized plasma membrane proteins was done by reacting the isothiocyanate-group of the isothiocyanatopropylsilica with the amino groups of the proteins.

The dry, "activated" carrier was washed with coupling buffer (borate-buffer [50 mM], pH 8.0; 4 mM CHAPS) and resuspended in aliquots of solubilized plasma membrane proteins. Binding was done by mixing the suspension at 4°C for 2 d using an end-over-end mixer. Subsequently, the remaining reactive groups were blocked by incubation with DME for 24 h at 4°C with continuous mixing. Control silicabeads were reacted in the same manner without plasma membrane proteins. Beads were washed three times with PBS, treated with ethanol (70%, 10 min), washed sequentially three times with PBS, DME, and DME/10% fetal calf serum (FCS), respectively, and added to the cells.

For the experiments in which plasma membrane proteins were added to the cells in soluble form, the proteins from the 4-mM differential extract were concentrated over a membrane filter (Amicon, PM 10) and resuspended in DME. The concentration step in DME was repeated twice and the solution sterilized by UV-irradiation for 15 min. Before the addition to the cells, FCS was added to a final concentration of 10%.

1. *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

Measurement of DNA Synthesis

5×10^3 cells in DME/0.5% FCS were seeded per well of a microtiter plate. After 24 h, silicabeads with bound plasma membrane proteins in DME/10% FCS were added. Controls received beads without bound membrane proteins. After 20 h, 0.25 µCi [³H]thymidine (New England Nuclear, Braunschweig, FRG) was added to each well and the cells were cultured for an additional 4 h. The cultures were processed for measurement of incorporated radioactivity as described (20).

Determination of Cell Growth

5×10^4 cells in DME/10% FCS were seeded in 35-mm culture dishes. Beads bearing plasma membrane glycoproteins were added as described above. After 3 and 7 d, cells were counted after trypsinization using a hemocytometer. In some dishes, the beads were removed at day 3 by repeated flushing and the culture was continued until day 7.

Galactosidase and NaIO₄ Treatment

Beads bearing a total of 0.12 mg protein were incubated for 12 h in 0.5 ml PBS with 5 U β-galactosidase at 37°C or in 0.5 ml acetate-buffer, pH 4.5, 0.05 M NaIO₄ at 4°C for 24 h. Control beads were treated in the same manner without galactosidase or NaIO₄. After treatment the beads were washed three times with PBS and processed as described above.

Pronase Treatment of Cells

Cells grown to confluency were washed twice with PBS and treated subsequently with 0.01 mg pronase (pretreated for 30 min at 65°C) in 0.1 M Tris-HCl, 1 mM CaCl₂, pH 8.0, and centrifuged; and the supernatant containing the released glycopeptides was removed. The pelleted cells were used for the preparation of 4-mM CHAPS extract as described above. The supernatant was further processed by the addition of 0.4 mg pronase/ml and incubated at 37°C. The addition of fresh pronase was repeated after 24, 48, and 72 h. Thereafter, the digest was heated at 100°C for 15 min, cooled on ice, and centrifuged (10,000 g, 20 min). The plasma membrane glycopeptides in the supernatant were coupled covalently to activated silicabeads as described above.

Protein Quantification

Protein was estimated according to Lowry et al. (10) using bovine serum albumin as standard.

Determination of Procollagen Type III

Procollagen type III was determined by radioimmunoassay (21). The test-kit was purchased from Behringwerke AG, Frankfurt, FRG (Marburg).

Results

Isolation of Plasma Membranes

The procedure adapted for the isolation of plasma membranes from human fibroblasts yielded ~0.2 mg membrane protein from 1×10^7 cells with a 20- and 10-fold enrichment of plasma membrane marker enzymes, alkaline phosphatase, and alkaline phosphodiesterase, respectively, compared with the homogenate (26).

Influence of Isolated Plasma Membranes on the Growth of Human Fibroblasts

The growth of human fibroblasts was shown to be inhibited by isolated plasma membranes in a concentration-dependent manner (26). The observed inhibition was greatly reduced when plasma membranes, isolated from cells treated previously with tunicamycin, were used. Tunicamycin is an antibiotic which blocks the first step in the pathway of the synthesis of the oligosaccharide portion of asparagine-linked

glycoproteins (2). Treatment of isolated plasma membranes before the addition to the test cells with trypsin had only minor effects on the degree of inhibition.

Binding of Proteins to Derivatized Silicabeads

For the reconstruction of the contact environment of cells we searched for a rapid, mild, and efficient immobilization of plasma membrane molecules, thus yielding a kind of artificial cell with respect to cell-cell contacts. Silicabeads can easily be handled, and the previously developed cross-linker, 3-isothiocyanatopropyltriethoxysilane, has the advantage that native silicabeads can be modified and that it reacts selectively with amino groups of proteins in neutral aqueous solutions. Silicabeads derivatized in the described manner, with covalently bound lectins, have been shown to be an excellent support for the separation of glycoproteins by high performance liquid affinity chromatography (9, 19). In the course of these studies we found that binding of glycoproteins to the lectins, bound covalently to isothiocyanatopropyl-silica, was not altered even after about 80 chromatographic runs, indicating that leakage of bound proteins is negligible.

Influence of Silicabeads on Cell Growth Rate

When beads without the cross-linker 3-isothiocyanatopropyltriethoxysilane were added to cells, a statistically insignificant (5-10%) reduction of growth rate was observed. The same was true for beads previously reacted with the cross-linker, with or without blocking of the isothiocyanate group by preincubation in DME (data not shown).

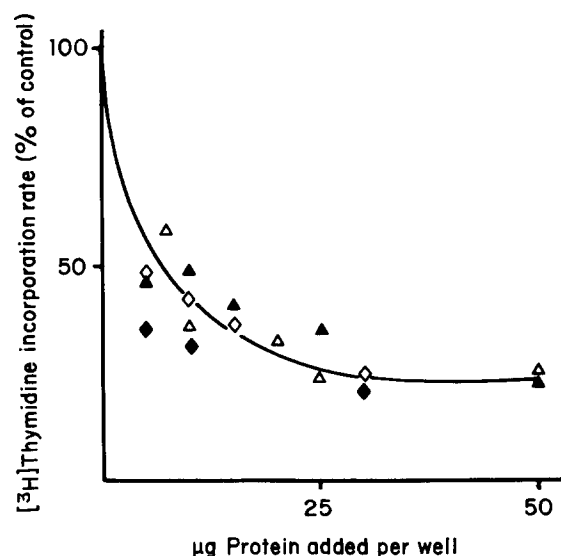


Figure 1. Inhibition of growth of sparsely seeded fibroblasts by immobilized plasma membrane glycoproteins. Different numbers of beads (bead number at 50 µg protein/well = 5×10^5), each bead bearing the same amount of bound proteins from the 4-mM extract of plasma membranes, were added to sparsely seeded cells and tested for their influence on incorporation of tritiated thymidine into DNA. Each point is the mean of six replicate determinations. The different symbols represent four different experiments. Control (100%): beads without bound plasma membrane proteins, reacted only with DME. The range of tritium counts incorporated into DNA in controls varied between 12,000 and 15,000.

Influence of Immobilized Plasma Membrane Glycoproteins on the Growth Rate

Sequential incubation of the beads bearing the isolated plasma membranes with increasing concentrations of CHAPS resulted in the preferential solubilization of the growth-inhibiting plasma membrane proteins (sevenfold enrichment, compared with a crude detergent extract) at a concentration of 4 mM CHAPS.

Further controls showed, that the specific activity of growth-inhibiting molecules increased in the following order: 16-mM extract of whole cells, 4-mM extract of whole cells, 16-mM extract of isolated plasma membrane, 4-mM extract of isolated plasma membranes (data not shown).

The growth rate of sparsely seeded cells was inhibited in a concentration-dependent manner by immobilized plasma membrane proteins of the 4-mM CHAPS extract, whereby the degree of inhibition depended on (a) number of beads added per cell and (b) the amount of protein coupled per bead. Fig. 1 shows the dependence of the growth inhibition on the number of beads added per cell. In this experiment, different numbers of beads were added to the cells, each bead bearing the same amount of protein. In Fig. 2, the number of beads added to the cells was constant, while the amount

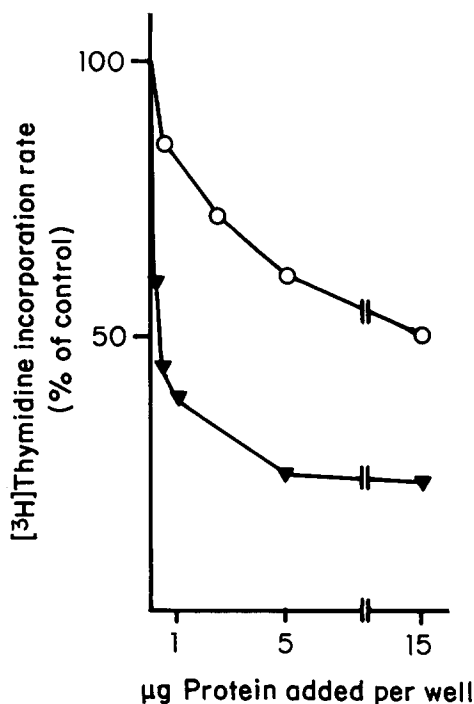


Figure 2. Inhibition of growth of sparsely seeded human fibroblasts by plasma membrane glycoproteins added in immobilized or in soluble form. (▼) Equal numbers of beads (5×10^5) bearing different amounts of proteins from the 4-mM extract of isolated membranes were added to sparsely seeded cells and tested for growth inhibitory activity. (○) Different amounts of proteins, in DME/10% FCS, from the 4-mM extract of isolated plasma membranes were added to sparsely seeded cells and tested for growth inhibitory activity. Values are expressed as percent [^3H]thymidine incorporation compared with the same system with beads without bound plasma membrane proteins or with medium without plasma membrane proteins.

of protein bound to the beads was varied. In this way, it was possible to determine the specific activity of the different preparations of solubilized proteins. The experiment depicted in Fig. 2 shows that $\sim 0.3 \mu\text{g}$ of bound plasma membrane proteins led to a 50% inhibition of growth. This value corresponds to the amount of membrane protein of $\sim 2.5 \times 10^3$ cells, calculated on the basis of purity and yield of membranes during purification. If the solubilized molecules were added in soluble form, 50% inhibition was achieved by the addition of $\sim 15 \mu\text{g}$ of plasma membrane glycoproteins of the 4-mM fraction to sparsely seeded cells (Fig. 2). For comparison, a 50% inhibition of DNA synthesis was obtained by the addition of 30 μg of plasma membranes to sparsely seeded cells (26).

Influence of Immobilized Plasma Membrane Glycoproteins on the Cell Growth

In addition to the influence of immobilized plasma membrane glycoproteins on the growth rate, their influence on cell growth was determined. In these series of experiments, the cell number in the presence or absence of immobilized plasma membrane glycoproteins was measured after different periods of culture. As shown in Fig. 3, the cell number in cultures in the presence of immobilized glycoproteins was reduced to 60–70% of that in control cultures. In addition, after the removal of the beads bearing the plasma membrane glycoproteins, after 7 d approximately the same cell number was found both in the control cultures as well as in the cultures grown for 3 d in the presence of immobilized glycoproteins. This indicates that the observed inhibition is fully reversible and is non-toxic.

Specificity of Inhibition of Growth by Immobilized Plasma Membrane Proteins

Previous studies (26) have shown that the inhibition of growth by glutaraldehyde-fixed cells was reversed when the cells to be fixed were pretreated with β -galactosidase or preincubated with lectins specific for galactose residues (e.g., *Bandeiraea simplicifolia*-lectin I, peanut agglutinin).

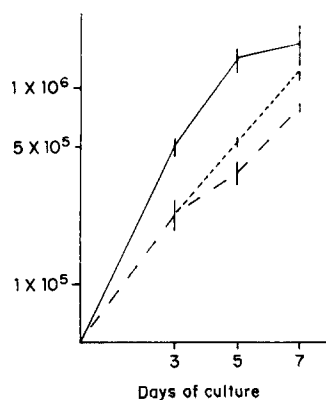


Figure 3. Inhibition of cell growth by immobilized glycoproteins as determined by cell number. 5×10^4 cells were seeded in DME/10% FCS in 35-mm dishes and cultured in the presence of beads (1×10^7) bearing plasma membrane glycoproteins from the 4-mM extract (---) or without membrane proteins (control: —). After 3, 5, and 7 d the cell number was determined in triplicate using a hemocytometer after the cells were

detached from the culture dishes with trypsin. In some dishes, the beads were removed at day 3 from cell cultures by repeated flushing with medium and the cells cultured for an additional 4 d (---). Each point is the mean \pm SD from three independent experiments.

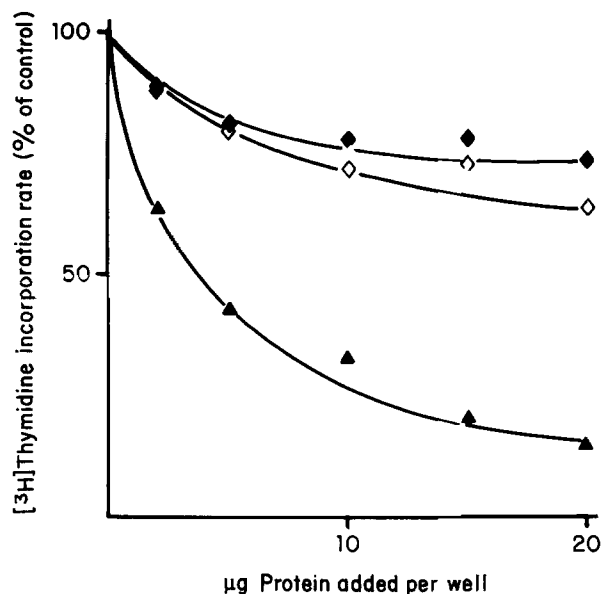


Figure 4. Effect of galactosidase or NaIO_4 pretreatment of immobilized plasma membrane glycoproteins on growth inhibitory activity. Beads bearing proteins from the 4-mM membrane extract were treated with β -galactosidase or NaIO_4 as described in Materials and Methods and tested in the proliferation assay. (▲) Beads without treatment; (◇) beads pretreated with galactosidase; (◆) beads pretreated with NaIO_4 . Controls for (▲): beads without bound proteins; for (◇): beads treated in the same manner, without galactosidase; for (◆): beads treated in the same manner, but without NaIO_4 .

Other enzymes or lectins (e.g., specific for fucose) were without influence on growth inhibition. We treated plasma membrane proteins covalently coupled to silicabeads with β -galactosidase or with NaIO_4 . As shown in Fig. 4, both treatments greatly reduced the growth inhibition. In contrast, treatment with α -galactosidase was without effect (data not shown).

Pronase treatment of the cells to be used for the isolation of the plasma membranes resulted in the release of glycopeptides which, when bound to activated silicabeads and added to sparsely seeded fibroblasts, were shown to inhibit the proliferation to a similar degree as intact plasma membrane glycoproteins. The remaining plasma membrane proteins without glycosidues, on the other hand, had only minor effects on the proliferation of fibroblasts (Fig. 5).

Procollagen Type III Synthesis

The increase in cell density in cultures of human fibroblasts is paralleled by an increased synthesis of procollagen type III (1). Fig. 6 shows the inverse correlation between the proliferation rate and procollagen type III synthesis. Using the method described by Sykes et al. (23), a correlation between the actual cell number and the ratio of collagen type III to type I has been found (data not shown). If the test system described above really imitates the situation occurring at high cell density, in addition to the reduction of the growth rate, an increased synthesis of procollagen type III should be induced by the addition of immobilized plasma membrane proteins to sparsely seeded cells. Fig. 7 shows the results of

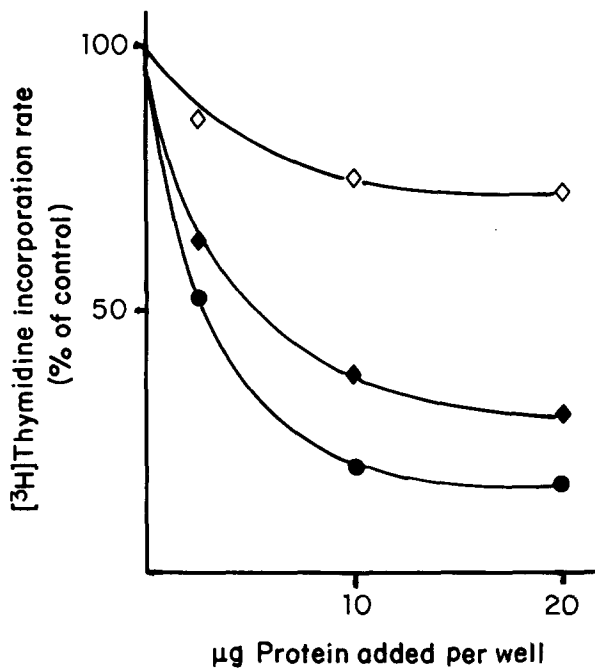


Figure 5. Influence of immobilized plasma membrane proteins (4-mM extract), isolated from cells pretreated with pronase, on the growth rate of sparsely seeded cells. Beads bearing plasma membrane proteins (4-mM extract) isolated from (●) untreated cells, or from (◊) cells pretreated with pronase. (◆) Glycopeptides released by pronase treatment from intact cells and immobilized onto derivatized silicabeads. Control (100%): beads without bound proteins.

preliminary experiments, giving evidence for a correlation between procollagen type III synthesis and growth inhibition, resembling the situation of cultures with increasing cell densities.

Discussion

The regulation of cellular proliferation is mediated by (a) supplemented humoral factors and (b) cell-cell interactions. When hormones and growth factors are available in optimal concentrations, the only growth-regulating mechanism seems to be the extent of cell-cell contacts, mediated by plasma membrane glycoproteins and sugar-specific receptors (5, 12, 17, 25). When the cells reach a critical density, thereby increasing the number of cell-cell contacts, signals are triggered into the cell leading to a decrease or cessation of growth. Transformed cells, on the other hand, are no longer susceptible to these specific cell-cell contacts, leading to uncontrolled growth. A recent report showed that transformed cells are obviously unable to establish these contacts due to a defect in the receptors of the glycoprotein(s) involved in the contact-dependent inhibition of growth (14). This finding is in good agreement with the theory of the clonal origin of tumors (16). According to Peterson and Lerch (17), the intracellular machinery, which processes the signal generated by the specific cell-cell contacts, is obviously intact in transformed cells.

In previous studies we have shown that growth of the fibroblasts used in these experiments was highly sensitive to cell density. High cell density could be imitated by the addi-

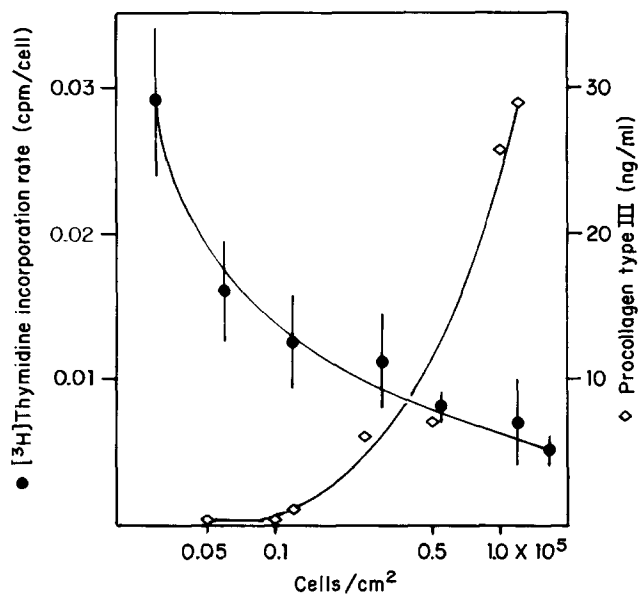


Figure 6. Synthesis of procollagen type III as a function of the cell density. Different numbers of cells (abscissa) were seeded in wells of a microtiter plate in DME without FCS and cultured for 24 h. Culture supernatants were removed, centrifuged, and tested by radioimmunoassay for procollagen type III peptide. Each point is the mean of 8 replicate determinations.

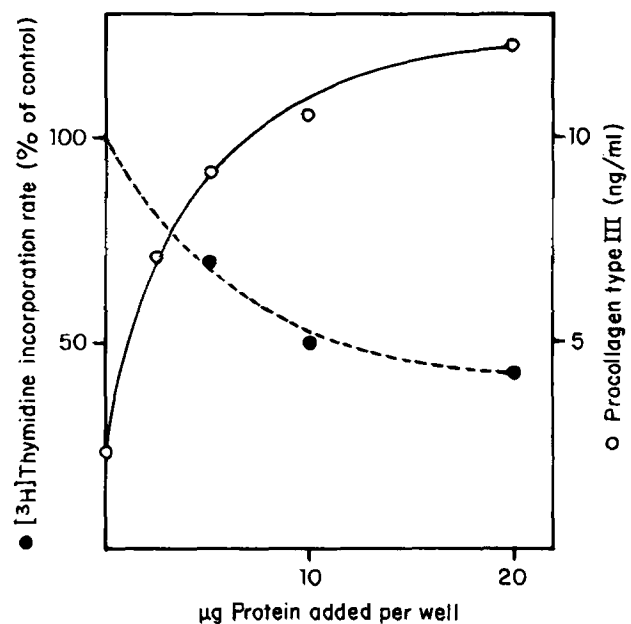


Figure 7. Synthesis of procollagen type III induced by the imitation of high cell density by the addition of immobilized plasma membrane glycoproteins to sparsely seeded cells in DME without FCS. The experiment was performed as described in Fig. 1, except that cells were cultured in medium without FCS. (○) Induction of collagen synthesis; (●) inhibition of growth by immobilized plasma membrane glycoproteins from 4-mM differential membrane extract. Control (100%): beads without bound plasma membrane glycoproteins, reacted with DME only. The range of tritium counts incorporated into DNA in controls varied between 2,300 and 2,900.

tion of glutaraldehyde-fixed cells or of isolated plasma membranes to sparsely seeded cells (26). In this report we have shown that high cell density could be successfully imitated by the addition of solubilized and immobilized plasma membrane molecules to proliferating fibroblasts. The beads alone act as a carrier of the plasma membrane glycoproteins and were themselves without effect on the cell growth. The lack of effect was also independent of whether the beads were bearing the cross-linker or not. Three lines of evidence argue for the involvement of distinct plasma membrane glycoprotein(s) in the contact-dependent inhibition of growth: (a) the growth inhibition caused by added plasma membranes was greatly reduced when the plasma membranes were isolated from cells previously treated with tunicamycin (26), an antibiotic which inhibits the synthesis of the oligosaccharide portion of asparagine-linked glycoproteins; (b) growth inhibition was reversed by NaIO_4 treatment of immobilized proteins; and (c) growth inhibition was reversed by treatment of the immobilized plasma membrane molecules with β -galactosidase. These data also argue against an unspecific influence of the hydrophobic proteins on the growth behavior of the fibroblasts. This possibility can also be excluded by the fact that plasma membrane molecules solubilized with 16 mM CHAPS showed a much lesser degree of inhibition of growth as the proteins solubilized with 4 mM CHAPS. Furthermore, membrane proteins isolated from cells after pronase treatment had only minor effects on the cell growth. The pronase treatment leads to the release of plasma membrane glycopeptides (the fraction in which the growth-inhibiting molecules have been found; Wieser, R. J., D. Renauer, and F. Oesch, manuscript in preparation), whereas the hydrophobic portion of the proteins inserted or spanning the plasma membrane still remain intact.

The degree of inhibition of growth is strongly dependent on the number of cell-cell and, therefore, of cell-plasma membrane glycoprotein contacts, respectively. The addition of isolated plasma membrane glycoproteins to the cells in soluble form obviously results in aggregation of the proteins, thereby reducing the number of possible interactions with the cells. This may also be the reason for the lower activity of the plasma membrane glycoproteins with regard to the inhibition of growth, when added to the cells in soluble form. A further explanation for the lower activity may be a reduced induction of rearrangement of plasma membrane molecules or a rapid internalization of the freely diffusible plasma membrane glycoproteins.

The involvement of glycoproteins (8, 11, 15, 18, 27) and of glycosaminoglycans (3) in the control of growth has also been shown by several other groups. The relatively slight effect of trypsin-treatment of isolated plasma membranes on their inhibitory potency (26) makes an involvement of glycosaminoglycans in the observed effects rather unlikely.

In addition to the induction of growth inhibition by immobilized plasma membrane glycoproteins, one further criterion characteristic for cultures at high cell density could be induced; namely, the synthesis of procollagen type III. The induction of specific enzyme activities characteristic for cultures at high cell density, by the addition of isolated plasma membranes to sparsely seeded hepatocytes, has previously been described (12).

In summary, this study further supports the hypothesis of growth regulation by specific cell-cell contacts via plasma

membrane glycoproteins as one of the reactants and shows that cell-cell contacts are successfully imitated by the addition of immobilized, specific plasma membrane glycoproteins to cultured cells.

The excellent technical assistance of Mrs. Rosario Heck is gratefully acknowledged.

This work was supported by the Deutsche Forschungsgemeinschaft (Wi 77/1-1).

Received for publication 21 August 1985, and in revised form 28 April 1986.

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