Influence of the carbohydrate moiety on the growth inhibitory activity and adhesiveness of 3T3 cell plasma membranes

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Summary. Treatment of 3T3 cell plasma membranes with glycosidase enzymes decreased their ability to inhibit cell growth and also decreased their binding to 3T3 cells. This suggests that carbohydrate is required for complete function of inhibitory activity and that inhibition is associated with membrane adhesion.
Key words. 3T3 cell; growth inhibition; glycosidase; plasma membrane.

Non-transformed cells undergo inhibition of growth (cell division) in vitro at high culture density whereas transformed cells exhibit no such growth regulation[1]. Cell-wounding experiments[2] first demonstrated the requirement of intercellular contact for growth inhibition. Whittenberger and Glaser[3] later reported that isolated plasma membranes added to subconfluent 3T3 cells caused inhibition of DNA synthesis. Growth inhibition by plasma membranes has since been demonstrated for other cell lines[4] and is accompanied by specific adhesion of the membranes to cells[5]. Plasma membrane-induced growth inhibition in 3T3 cells is mediated by integral membrane proteins[6, 7], and in other cell lines membrane-derived glycopeptides[8] and sialic acid-containing membrane proteins[9] have been shown to inhibit growth.

Although the contribution of carbohydrate to the function of glycoproteins remains controversial, evidence implicates its involvement in cellular adhesion. For example, cell-substrate adhesion is altered by removal of cell-surface carbohydrate by either glycosidase enzymes[10] or tunicamycin[11]. It is not as apparent, however, whether membrane-associated carbohydrate plays a similar role in mediating density-dependent growth inhibition.

The objective of this study is to determine more precisely the contribution of carbohydrate to the function of the growth inhibitory activity of 3T3 cell plasma membranes. It was found that selective removal of carbohydrate moieties causes a reduction in growth inhibition accompanied by a reduction in the adhesion of membranes to cells.

Materials and methods. Swiss 3T3 cells were grown in 100 × 15 mm plates (Flow) at 37 °C in a 5% CO_2/95% air atmosphere in Dulbecco's modified Eagle's Medium (DME) containing 10% fetal bovine serum (Flow) or 10% calf serum (Sterile Systems), penicillin (5000 U/ml), streptomycin (5000 meq/ml) and 2 mM L-glutamine. A plasma membrane-enriched fraction, as determined by phosphodiesterase activity[3], was prepared from 1-day post-confluent cultures of 3T3 cells by the method of Whittenberger and Glaser[3]. Membranes were frozen in 10% glycerol at −70% for up to 60 days before using. The DNA-synthesis-inhibiting activity of the membranes was unchanged after freezing. To remove terminal monosaccharide residues, membranes were incubated with 25 mU of the (Sigma) exoglycosidases neuraminidase (from *Clostridium*) at optimum pH 5.0, α-fucosidase (bovine epididymus) at pH 6.5, or α-mannosidase (jack bean) at pH 4.5 for 30 min at 37 °C. Membranes were also treated with 20 mU of endoglycosidase D (Miles) in the presence of 25 mU neuraminidase, 12 mU β-galactosidase (jack bean) and 5 mU N-acetylglucosaminidase B (bovine epididymus) at pH 6.0 for 1 h to remove larger oligosaccharide moieties. Control membranes for each enzyme tested were incubated under identical conditions (pH, temperature) but without enzyme. After enzyme treatment all membranes were washed and centrifuged twice to remove enzyme and free sugars. Exoglycosidase activities were measured by the p-nitrophenol pyranoside reaction[12] except for neuraminidase, which was measured by release of free sialic acid from mucin[13]. Endoglycosidase activity was determined by the method of Muramatsu[14]. Glycosidases were assayed for protease activity by established procedures[15]. Membranes were treated with periodate by the method described by Raben et al.[6]. To measure rates of DNA synthesis, cells were grown in DME with 10% serum for 24 h, then starved for 24 h in DME with 0.5% serum. Membranes were added to the cells and incubated at 37 °C for 4 h. Cells were washed and incubated with DME with 10% serum for 18 h. Rates of cell growth were measured by determining incorporation of [^{3}H]-thymidine into DNA during a 1-h pulse labeling period[7]. Results were presented as percent stimulation over background with cells incubated in DME with 10% serum alone considered 100% maximal stimulation. Inhibition is determined as percentage of the maximal stimulation. Adhesion of 3T3 and SV3T3 membranes to 3T3 cells was determined by labeling membranes with ^{125}I by the chloramine T method[16] and quantification by a method adapted from Lieberman et al.[5].

Results. Treatment of 3T3 cell plasma membranes with glycosidase results in decreased growth inhibitory activity, although the degree of reduction was not the same for different enzymes (fig. 1). Removal of terminal monosaccharides with neuraminidase and α-mannosidase reduced growth inhibition only slightly (25% and 34% respectively) whereas treatment with α-fucosidase decreased in-

hibitory activity to a much greater extent (100%). Treatment of membranes with neuraminidase, N-acetylglucosaminidase and β-galactosidase together (fig. 1, bar F), which removes the terminal three residues which inhibit endoglycosidase, reduced membrane-associated inhibition more (76%) than did removal of single residues by neuraminidase or mannosidase, and with the addition of endoglycosidase to these three exoglycosidases (fig. 1, bar G) inhibitory activity was further reduced (98%). Oxidation of carbohydrate residues with periodate decreased inhibitory activity by 62%.

To determine whether carbohydrate affects cell-membrane adhesion, the binding of glycosidase-treated plasma membranes to 3T3 cells was examined. The results (fig. 2) show that removal of carbohydrate from 3T3 cell membranes (by fucosidase and endoglycosidase) significantly ($p < 0.01$) reduced their binding to cells. The decrease by glycosidases on the binding of membranes to cells (37%) was not as great as its effect on growth inhibition. To determine if the effect on adhesion was specific for 3T3 cell plasma membrane carbohydrate, the binding of SV3T3 cell plasma membranes to 3T3 cells was also examined. The binding of untreated SV3T3 cell membranes was significantly lower ($p < 0.01$) than that of untreated 3T3 cell membranes. In addition, there was no observed decrease in binding of SV3T3 cell membranes after treatment with glycosidase (fig. 2).

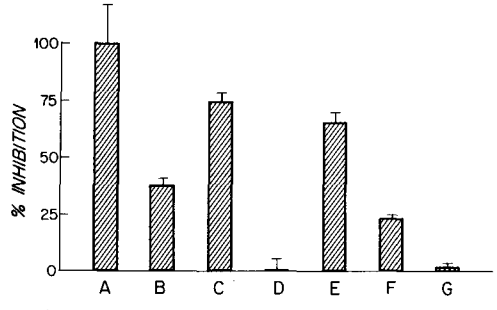

Figure 1. Effects of glycosidase and periodate on the growth inhibitory activity of 3T3 cell plasma membranes. Membranes were digested with periodate or glycosidase enzymes (each at their optimum pH) and then added to subconfluent 3T3 cells (125 μg membrane protein/2×10^5 cells). Rates of DNA synthesis were determined 18 h later after addition of DME with 10% serum and growth inhibition determined. Inhibition by treated membranes is expressed as percentage of the maximal inhibition observed for untreated (control) membranes. A, control; B, periodate; C, neuraminidase; D, fucosidase; E, mannosidase; F, neuraminidase + N-acetylglucosaminidase + β-galactosidase; G, endoglycosidase + neuraminidase + N-acetylglucosaminidase + β-galactosidase. Controls averaged 5120 ± 1691 CPM. Observed differences except for E are significant ($p < 0.01$ for D, F, G; $p < 0.025$ for B, C).

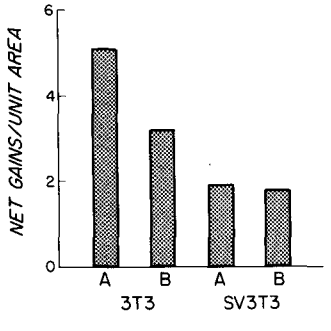

Figure 2. Binding of [^{125}I]-labeled 3T3 and SV3T3 cell plasma membranes to 3T3 cells. Untreated and glycosidase-treated (α-fucosidase + endoglycosidase) membranes were incubated with cells for 5 h. Cells were washed, fixed and autoradiographed as described in 'Materials and methods'. Net grains per unit area were determined by subtracting the average number of grains/unit area over background (areas with no cells) from random areas directly over cells. A, control membranes; B, glycosidase-treated membranes. Observed differences are significant ($p < 0.01$).

Discussion. The results indicate that carbohydrates associated with the 3T3 cell plasma membrane are required for optimum function of membrane-induced growth inhibition. Fucosyl residues appear to be important for inhibitory activity function (fig. 1), although this effect cannot be generalized for other terminal monosaccharides since lesser reduction in inhibition was observed by removal of sialic acid and mannose residues. Removal of multiple residues with more than one exoglycosidase, or of larger oligosaccharide moieties with endoglycosidase caused a greater reduction in inhibition than did removal of single terminal monosaccharides except for fucose. It is unlikely that the present results were due to protease contamination, because: 1) the glycosidases used contained no detectable protease activity by the methods used[15] and 2) previous results[7] have shown that the inhibitory activity of 3T3 cell plasma membranes is negatively affected only by protease concentrations (750 μg/mg membrane protein) much greater than would be present as contaminant in commercially prepared glycosidases at the concentrations used in the present experiments.

The reduction of growth inhibition by glycosidase enzymes is accompanied by a decrease in the binding of membranes to 3T3 cells (fig. 2). These results are consistent with the study by Lieberman et al.[5] who showed that factors that decrease membrane-associated inhibition (heat denaturation, high serum concentration) also reduce membrane adhesiveness. Thus membrane carbohydrate positively affects growth inhibition by increasing specific cell-membrane adhesion. The requirement of adhesion for the full expression of the growth inhibitory activity of membranes is specific for 3T3 cells, because SV3T3 cell plasma membranes, which do not inhibit cell growth[3], bind less to cells than do 3T3 cell membranes and do not exhibit decreased binding after glycosidase treatment. The unique characteristics of 3T3 cell plasma membrane carbohydrate that enable growth inhibition and adhesion are thus lost or altered upon transformation. The observed effect of removal of plasma membrane-associated carbohydrates on both adhesion and growth inhibition implicates some functional relationship, either direct or indirect, between the membrane glycoproteins mediating the two cellular processes.

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Detection of the *H-RAS* oncogene in human thyroid anaplastic carcinomas

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Summary. We have transfected high-molecular-weight DNA from human thyroid carcinomas into murine 3T3 cells. As a result we identified several foci of morphologically distinct transformed cells in each of the tumour DNA transfected cultures. After a total of three rounds of transfection, the transformed cells were shown to form tumours in nude mice. Southern blot analysis of DNA prepared from third-round transfectants demonstrated the presence of human Alu repetitive sequences and, after hybridization with probes for known oncogenes, indicated the presence of the human *H-RAS* oncogene in 3T3 cells transfected with three out of four anaplastic carcinoma DNA samples. It appears therefore that activation of *RAS* genes may be an important event in the development of the anaplastic thyroid tumours.
Key words. Anaplastic carcinoma; thyroid; *H-RAS* oncogene; transfection.

Thyroid cancer is the fourth most common form of neoplastic disease occurring in women in Saudi Arabia, accounting for 5.7% of all cancers observed in females[1]. The reason for this high incidence is unknown and does not appear to be related to goitre. Thyroid cancer is therefore of particular interest in this region and, as oncogenes have gained considerable notoriety over the past few years for their association with neoplastic disease[2–4], we decided to investigate which of the proto-oncogenes are activated in thyroid tumour tissue. In particular, we were interested in determining whether we could detect the presence of a dominant transforming gene in any of the thyroid tumour DNA samples by transfecting tumour DNA into murine 3T3 cells.

The development of the technique for DNA transfer into animal cells[5,6] has led to the discovery of cellular transforming genes (in human tumour cells) because of their ability to induce foci of morphologically distinct transformed murine 3T3 cells[7]. A number of new oncogenes have been discovered by this method, including *N-RAS*[8], *MET*[9] and *MAS*[10]. Furthermore, this method has also allowed the transforming potential of known oncogenes to be demonstrated[7]. To increase the sensitivity of the 3T3 transfection technique, cotransfection with a selectable marker has also been used in conjunction with tests of tumourigenicity of the transfected 3T3 cells in nude mice[11]. It is a modification of this latter approach that we have used in attempting to detect the presence of dominant transforming genes in thyroid tumours.

Materials and methods. Each tumour was snap-frozen in liquid nitrogen immediately after surgical removal, and then stored at −70 °C until required. The frozen tumour tissue was ground under liquid nitrogen and high-molecular-weight DNA was prepared either from this material, or from 3T3 cell pellets, by using the proteinase K method[12].

Murine 3T3 fibroblasts were routinely grown in Dulbecco's modified Eagles medium, supplemented with 10% (v/v) newborn calf serum, in a humidified CO_2 incubator at 37 °C. During propagation of the cultures, the cells were always harvested before they became confluent. In each transfection experiment 2×10^5 cells were seeded into each 90-mm petri dish, and left overnight before transfection. Usually 20 dishes were used for each test.