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Original Article

Changes in surface glycoconjugates in adhesion-defective variants of P19 embryonal carcinoma cells

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ABSTRACT Embryonal carcinoma cells defective in their ability to adhere to tissue culture dishes were isolated from mutagenized P19X1 and P19S1801A1 cells. Three independently isolated variants were analyzed for their morphology, surface properties and ability to differentiate *in vitro*. Two of the mutant cell lines expressed similar amounts of stage-specific embryonic antigens TEC-1, TEC-4 and Thy-1 as parental cells, whereas all three showed significant reduction in the expression of uvomorulin as determined by a direct radioantibody binding assay. Variant cells exhibited a decrease in their ability to aggregate in media with or without CA^{2+} and were unable to form compact aggregates when cultured for two days in complete culture media. In the presence of retinoic acid variant cells formed aggregates which exhibited significantly lower frequency neuron formation after transfer to tissue culture dishes. The combined data indicate that the adhesion-defective phenotype of P19-derived cells is in part the result of a reduced surface expression of uvomorulin.

KEY WORDS: *embryonal carcinoma, surface antigen, uvomorulin, embryoglycan*

Introduction

Cell surface molecules play an important role in the process of embryonic development. Mouse embryonal carcinoma (EC) cells, which share a number of properties with early embryonic cells (Martin, 1980; Kimber, 1990) have a key role in elucidating what surface structures are involved in cell-to-cell and cell-to-surface adhesion and compaction. Monoclonal and polyclonal antibodies produced after immunization with EC cells identified several surface components which are directly involved in cell adhesion. One of the best characterized surface components important for cell adhesion and compaction is uvomorulin (E-cadherin), a member of the family of Ca^{2+}-dependent cell adhesion molecules (Kemler *et al.*, 1977; Ringwald *et al.*, 1987; Takeichi, 1988; Nose *et al.*, 1990). Other components such as carbohydrates (Grabel *et al.*, 1979; Nomoto *et al.*, 1986; Dráber *et al.*, 1988) are less well-characterized although their strict developmental regulation suggests their importance in intercellular communications (Shevinsky *et al.*, 1982). This is supported by recent data suggesting that a stage-specific embryonic antigen 1 (SSEA-1, lacto-N-fucopentaose III) is responsible for cell recognition in 8-cell mouse embryo (Bird and Kimber, 1984; Fenderson *et al.*, 1984), and that carbohydrate-carbohydrate interactions may be involved in such recognition (Eggens *et al.*, 1989a,b).

In this laboratory we have isolated several cell surface variants defective in the expression of lectin receptors (Dráber and Stanley, 1984) or stage-specific embryonic antigens (Dráber and Vojtisková, 1984; Dráber and Maly, 1987). Although the variant cells exhibited significant changes in their surface carbohydrate make-up, they resembled the parental EC cells in a number of properties, including cell-to-substrate adhesion and formation of aggregates after several days in culture. So far we have identified only one F9-derived mutant cell line, W3, resistant to a lectin from *Ricinus communis* which adhered poorly to tissue culture dishes and formed less compact aggregates (Dráber and Stanley, 1984). All our mutants were selected for resistance to cytotoxic plant lectins or anti-SSEA-1-ricin conjugates. In this paper we describe the selection and the properties of P19-derived cells that were selected for their inability to attach to tissue culture dishes. These cells expressed variable amounts of surface carbohydrate epitopes but all of them showed a significant decrease in the expression of uvomorulin.

Results

Selection of mutants

Preliminary experiments indicated that unmutagenized P19X1 and P19S1801A1 cell suspensions (2.5 x 10^7 cells; relative cloning efficiency 45%) did not contain non-adherent cells. In further

Abbreviations used in this paper: CMF medium, Ca^{2+} and Mg^{2+} free medium; BSA, bovine serum albumin; EC, embryonal carcinoma; EMS, ethyl methane sulfonate; mAb, monoclonal antibody; MNNG, N-methyl-N'-nitro N-nitrosoguanidine; PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 7.2; RIA, radioantibody binding assay; UMt, uvomorulin.

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TABLE 1

SELECTION OF ADHESION-DEFECTIVE EC CELLS

Exp. No.	Parental cells	Mutagen (μg/ml)	No. of cells used for selection	Adhesion-defective lines obtained[a]
1.	P19X1	EMS (200)	3×10^7	P19XAd$^-$B.2 P19XAd$^-$4.1
2.	P19S1801A1	EMS (200)	5×10^6	–
3.	P19S1801A1	EMS (100)	3×10^7	–
4.	P19S1801A1	MNNG (2)	2.4×10^7	–
5.	P19S1801A1	MNNG (1)	3×10^7	P19SAd$^-$9.0

[a]0.1×10^6 EC cells/ml were treated with the indicated concentrations of mutagen EMS or MNNG as described in Materials and Methods. After 7 day-expression period the cells were plated on tissue culture dishes at a concentration of 0.12×10^6 cells/ml. Every third day the cell culture supernatant containing weakly adherent and non-adherent cells was harvested by pipetting, washed and transferred to a new dish. The same procedure was repeated for two months. Three independent adhesion-defective phenotypes were identified.

experiments we therefore used mutagen-treated cells. The concentration of mutagens, 0.2 mg/ml of EMS or 2μg/ml of MNNG, caused approximately a 60-80% reduction in cell cloning efficiency. We succeeded in isolating three mutant EC cell lines defective in their ability to adhere to cell culture surfaces and to form compact aggregates: P19XAd$^-$B.2, P19XAd$^-$4.1 and P19SAd$^-$9.0 (Table 1). These clones weakly attached to the plastic and easily came off into culture medium after shaking of the culture dishes. Loss of adhesive properties of mutant cells could be demonstrated by counting the number of floating and weakly adhering cells at various time intervals of cultivation. Thus, 50% of parental cells (P19X1 and P19S1801A1) adhered to tissue culture surface approximately after $1^{1/2}$ h of cultivation, whereas 50% of mutant cells P19XAd$^-$B.2, P19XAd$^-$4.1 and P19SAd$^-$9.0 required $1^{1/2}$ h, 24 h and >48 h, respectively (Fig. 1). When cultured in tissue culture dishes parental cells (Fig. 2a) formed monolayer of cells spread on the tissue culture surface. P19XAd$^-$B.2 mutant cells grew under the same condition as spherical colonies of weakly interacting cells with easily distinguished individual cell boundaries (Fig. 2b). The morphological difference between P19XAd$^-$4.1 mutant cells and parental cells was less significant. These cells spread on tissue culture surfaces but they did not form close cell-to-cell contacts (Fig. 2c). The most interesting isolated mutant cell line – P19SAd$^-$9.0 – was characterized by a lack of close cell-to-plastic and cell-to-cell interactions (Fig. 2d). The cells grew singly or in groups of several spherical cells, suspended in the culture medium. More than 50% of the cells were floating in the medium even after 1 day in culture (see Fig. 1).

Cell surface properties of mutant cells

It has been previously shown that monoclonal antibodies TEC-01, TEC-04 and 1aG4 reacted with undifferentiated P19 EC cells (Dráber and Pokorná, 1984; Dráber *et al.*, 1989a, b). The data obtained in direct RIA indicated that P19XAd$^-$4.1 and P19SAd$^-$9.0 cell lines expressed similar amounts of the stage-specific embryonic antigens TEC-1, TEC-4 and Thy-1 as the parental cells do (Fig. 3). The finding that P19XAd$^-$B.2 mutant cells bound less of these mAbs than parental cells may be related to a differentiation of these cells in the course of selection: the binding of TEC-01and TEC-04 antibodies to P19XAd$^-$B.2 cells was however significantly higher than their binding to L-M (TK$^-$) fibroblasts used as a negative control. All three mutant cell lines exhibited a significant decrease in the expression of UMt as detected by binding of the DECMA-1 antibody. As an internal control we also used the P19XT.1.1 cells defective in the expression of embryoglycan (Dráber and Maly, 1987). These cells did not bind TEC-01 antibody and bound similar amounts of TEC-04 and 1aG4 antibodies as the parental cells. It has been shown that P19XT.1.1 cells do not exhibit any significant defects in their ability to form aggregates when cultured for several days on bacteriological-grade dishes (Dráber and Maly, 1987). This probably corresponds to the finding presented in Fig. 3 that they bind the same amount or even more DECMA-1 mAb than parental P19X1 cells.

Aggregate-forming properties and differentiation of mutant cells

To determine whether mutant cells differ from parental cells in their ability to form aggregates we analyzed aggregation of cells after their cultivation in agar-coated dishes which prevented cell interactions with tissue culture substrate. Fig. 4a shows that parental P19X1 cells formed big spherical compacted aggregates. Similar aggregates were observed when P19S1801A1 cells were analyzed (not shown). All mutant cell lines produced smaller aggregates which were less compacted and had irregular shapes (Fig. 4b-d). A number of mutant cells did not form aggregates and were floating in the culture medium. The most dramatic defect in their ability to aggregate was observed with P19SAd$^-$9.0 mutant cell line (Fig. 4d). These cells were therefore analyzed in a short-term

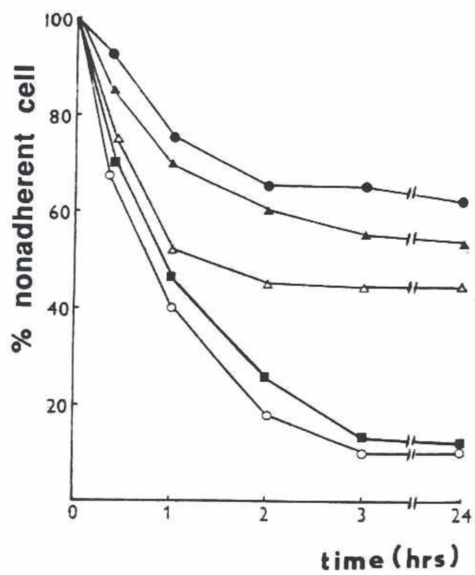

Fig. 1. Adhesive properties of parental and mutant cells. *Parental cells [P19X1 (○–○) and P19S1801A1 (■–■)], and mutant cells [P19XAd$^-$B.2 (△–△), P19XAd$^-$4.1 (▲–▲), and P19SAd$^-$9.0 (●–●)] were incubated in tissue culture dishes in tissue culture medium and at various time intervals the dishes were mixed and number of floating cells was determined. Average values of three experiments are shown.*

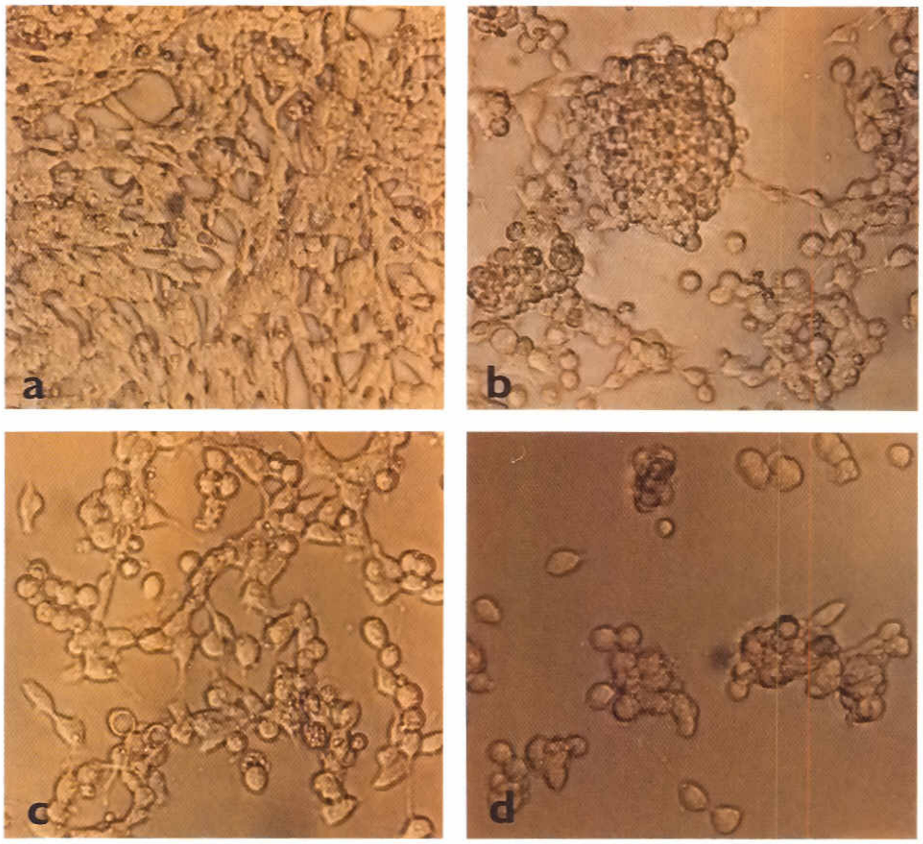

Fig. 2. Photomicrographs of parental and mutant cells. *The cells were cultured for three days in culture medium in tissue culture dishes. Morphology of P19X1 parental cells* **(a)**, *P19XAd$^-$ B.2 cells* **(b)**, *P19XAd$^-$4.1 cells* **(c)** *and P19SAd$^-$ 9.0 cells* **(d)**. *Magnification x200.*

aggregation assay. In the presence of Ca^{2+} the mutant cells formed small aggregates containing significantly fewer cells than those of P19X1 cells (Fig. 5a). In CMF medium the aggregation-forming capacity of the mutant cells was almost suppressed, whereas in parental cells significant numbers of aggregates were formed, although less frequently than in Ca^{2+}+Mg^{2+}supplemented medium (Fig. 5b). These data indicate that both Ca^{2+} and Mg^{2+} dependent and independent surface components are important for an early aggregation of P19-derived cells. In the presence of retinoic acid, aggregation of parental P19X1 and P19S1801A1 cells in agar coated dishes for several days and subsequent transfer of the aggregates to tissue culture dishes resulted in the formation of neuron-like cells (Jones -Villeneuve *et al.*, 1982; McBurney *et al.*, 1988; Fig. 6a). It has been suggested that a three-dimensional structure and cell compaction was essential for neuronal differentiation (Jones-Villeneuve *et al.*, 1982). In further experiments we therefore analyzed neuronal-like differentiation of our mutant cell lines. We have found that the formation of less compacted aggregates of P19XAd$^-$B.2 and P19XAd$^-$4.1 cells resulted in a significant decrease of aggregates forming neuron-like processes; thus, when P19X1 and P19S1801A1 cells were used more than 95% of aggregates formed neuron-like processes, whereas when P19XAd$^-$ B.2 and P19XAd$^-$4.1 cells were used, neuron-like processes were formed in only 37% and 40% aggregates, respectively (Fig. 6b, c). P19SAd 9.0 cells were not able to form compact aggregates and when these cells were treated with retinoic acid and transferred to tissue culture dishes they neither adhered to tissue culture substrate nor formed aggregates containing neuron-like processes.

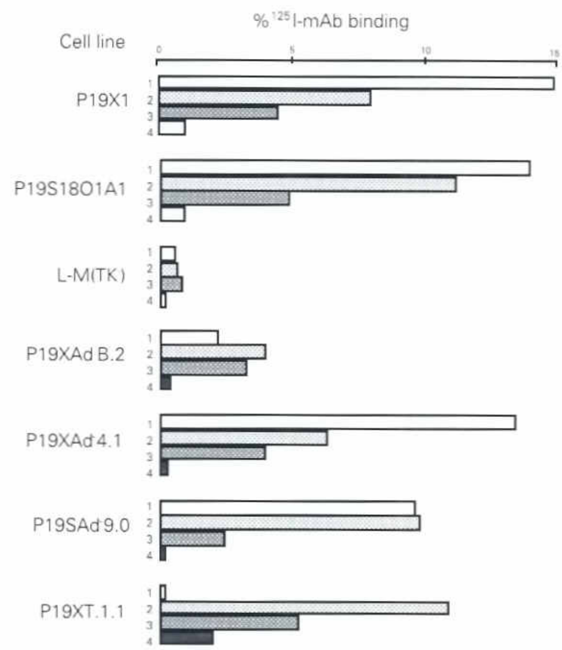

Fig. 3. Binding of [^{125}I]-labeled monoclonal antibodies TEC-01 (1), TEC-04 (2), 1aG4 (3) and DECMA-1 (4) to parental, mutant and control cells. *2.5 x 10^5 cells were incubated with [^{125}I]-labeled antibody (10^5 cpm) and percentage of radioactivity bound was determined in direct RIA. The results are averages of three experiments performed in triplicate.*

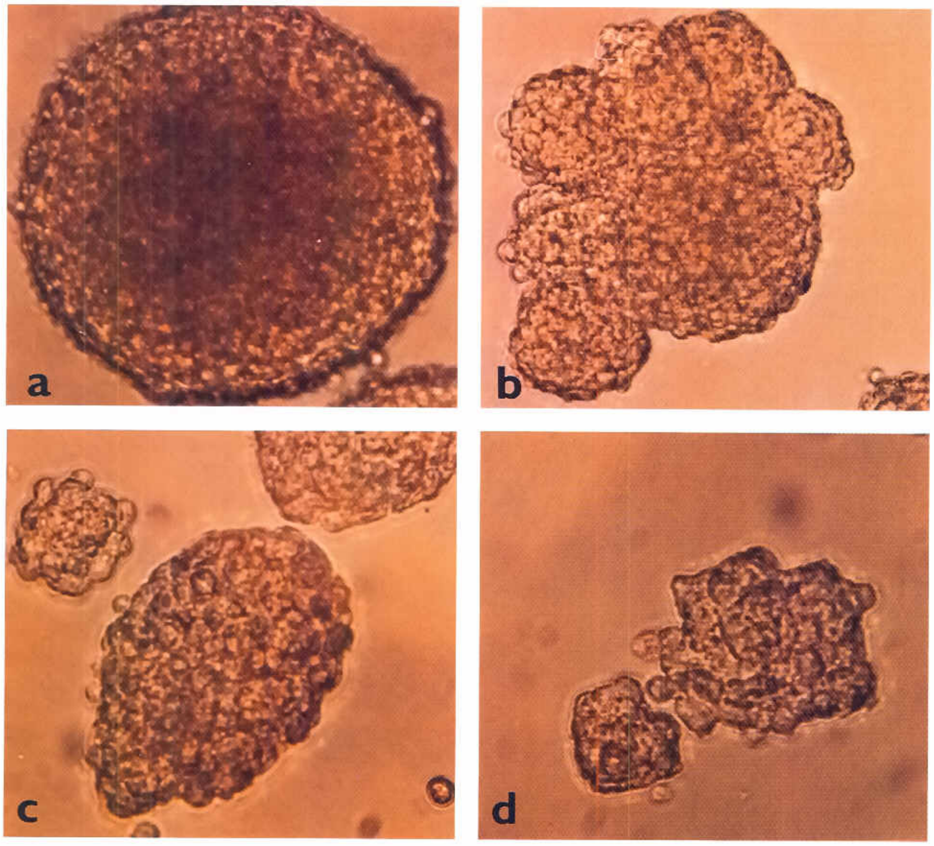

Fig. 4. Aggregates of the cells formed after 3 days in culture on 1.5% agar. *Spherical tightly compacted aggregates of P19X1 cells* **(a)**; *irregular loosely compacted aggregates of P19XAd$^-$B.2* **(b)**; *P19XAd$^-$4.1* **(c)** *and P19SAd$^-$9.0* **(d)** *cells.*

Discussion

We have succeeded in isolating three adhesion-defective cell lines derived from P19 cells. These cells were isolated by repeated harvesting of non-adherent or weakly adherent cells after treatment with mutagens. Since 2.5 x 10^7 unmutagenized cells (cloning efficiency 45%) yielded no non-adherent cells the frequency of spontaneous mutations was less than 10^{-7}. All mutant cell lines differed from the parental P19X1 or P19S1801A1 cells not only in their ability to adhere to tissue culture dishes but also in their surface antigen make-up, their ability to aggregate in a short term assay in the presence of Ca^{2+}, their ability to form aggregates after several days of cultivation on agar surface in the presence or absence of retinoic acid, and in their ability to form aggregates with neuron-like processes. One mutant cell line, the P19XAd$^-$B.2, was characterized by reduced expression of all three stem cell antigens analyzed: the TEC-1, TEC-4 and Thy-1.2. Because all three antigenic markers are developmentally regulated (Dráber and Pokorná, 1984; Dráber *et al.*, 1989a, b), it seems that the reduced expression reflects a shift to more differentiated cells. This shift is probably unrelated to the defect in adhesion because differentiated derivatives of P19 cells usually adhere to tissue culture dishes more than the prenatal cells do. Two other adhesion-defective variants P19XAd$^-$4.1 and P19SAd$^-$ 9.0 have an antigenic make-up that is similar to that of parental cells. All adhesion-defective cell lines, however, expressed significantly reduced amounts of UMt. Although we have no direct evidence it is very likely that the decrease in the expression of UMt is at least in part responsible for adhesion- and compaction-defective phenotype. It has been shown that this molecule plays an important role in cell adhesion and compaction (Takeichi, 1998).

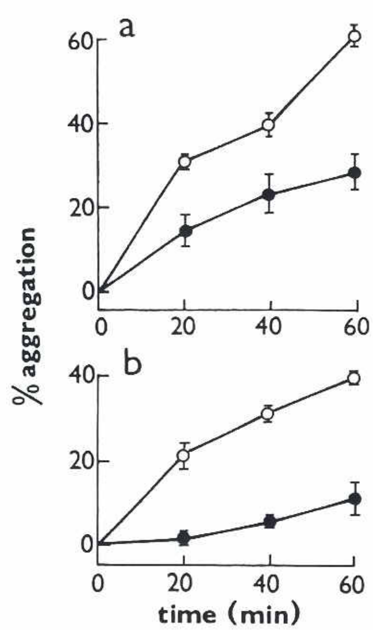

Fig. 5. Cell aggregation in the presence (a) or absence (b) of Ca^{2+}. *The P19X1 (○–○) and P19SAd$^-$ 9.0 (●–●) cells were incubated in CMF medium supplemented with Ca^{2+} + Mg^{2+}* **(a)** *or in CMF medium* **(b)** *as described in Materials and Methods. The aggregation was calculated as the percentage reduction in total particle number from time zero. The results represent the mean ± SD of the values obtained from three experiments performed in triplicate.*

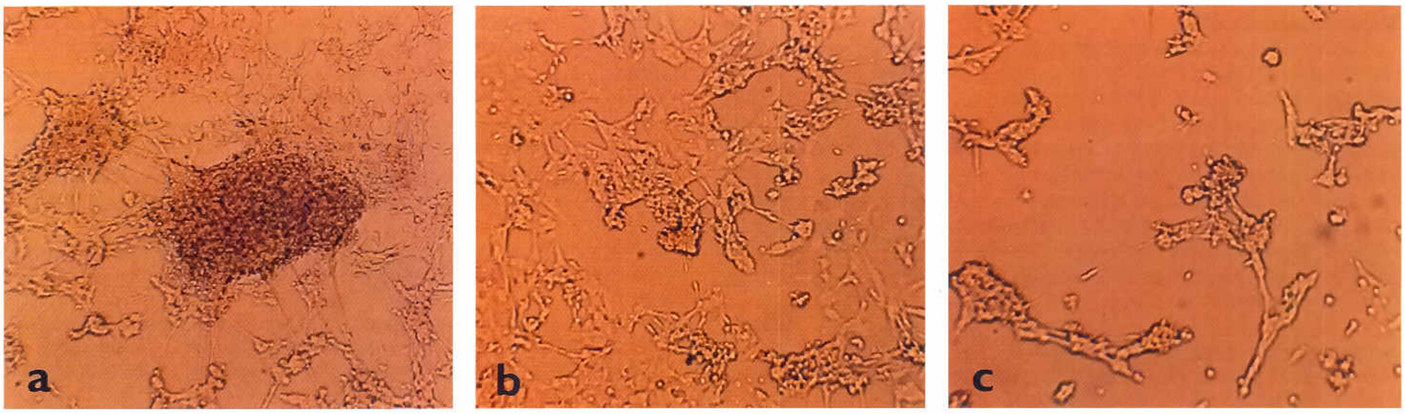

Fig. 6. Photomicrographs of cells from aggregates formed by parental and mutant cells. *P19X1* **(a)**, *P19XAd⁻B.2* **(b)** *and P19XAd⁻4.1* ***(c)*** *cells were induced to differentiate in the presence of 0.5 μM retinoic acid for 4 days in culture on 1.5% agar, then transferred to tissue culture dishes and further incubated for 3 days. Magnification x200.*

Furthermore, recent data on analysis of adhesion-defective variants indicated that most of them exhibited a defect in UMt. Thus, Adamson *et al.* (1990) found that adhesion-defective F9 cells expressed 40-50% of normal levels of UMt and Littlefield and Whitehouse (1990) reported that the compaction-defective variant of H6 cells exhibited a significant decrease in UMt expression. The key role of UMt in cell-to-cell and cell-to-substrate adhesion is also implied by our finding that P19XT.1.1 cells defective in the expression of embryoglycan and several stem cell carbohydrate epitopes are similar to parental cells in their ability to adhere to substrate and to form compact aggregates after several days in culture (Dráber and Maly, 1987). As shown in this paper these cells have a normal level of UMt.

Recent data indicated that multivalent carbohydrate-carbohydrate interactions mediated by bivalent cations may play a major role in the initial step of specific recognition between cells (Fenderson *et al.*, 1984; Eggens *et al.*, 1989a,b). Whether these interactions play a role in adhesion-defective phenotypes of our mutant cells remains to be established. Mutant P19 cells described in this paper were unable to form compact aggregates and were characterized by reduced number of aggregates containing neuron-like processes after treatment with retinoic acid. The most significant defect in all the properties analyzed was observed in P19SAd⁻9.0 cells. These cells were defective not only in cell-to-substrate adhesion but also in cell-to-cell adhesion. Interestingly, although these cells exhibited decreases in the expression of UMt, their cell-to-cell adhesion was dependent on Ca^{2+} and Mg^{2+}. This suggests that either a small amount of UMt expressed on the cell surface was crucial for the observed cell-to-cell interactions or that other Ca^{2+} and Mg^{2+}-dependent mechanisms play an important role in this phenomenon. For example Le^x-Le^x interactions are also dependent on the presence of Ca^{2+} and Mg^{2+} ions (Eggens *et al.*, 1989a). However, the ability of cells with reduced expression of SSEA-1 (P19XAd⁻B.2 and P19XT.1.1) to form aggregates points to complexity of adhesion phenomena in this system. The adhesion-defective mutants together with previously isolated carbohydrate-defective mutants may be a valuable material for further analyses of adhesion during embryonic development.

Materials and Methods

Cell lines and culture conditions

EC cell lines employed in the present experiments are subclones of the P19 cell line (McBurney and Rogers, 1982). An ouabain-resistant and 6-thioguanine-resistant subclone P19S1801A1, was generously provided by M.W. McBurney of the University of Ottawa. The P19X1 cell line was derived in this laboratory from a tumor obtained in a C_3H/Di mouse after subcutaneous injection of P19 cells (Dráber and Maly, 1987). P19XT.1.1 is an embryoglycan-defective mutant EC cell line derived from P19X1 (Dráber and Maly, 1987). L-M (TK⁻) is a transformed murine fibroblast cell line (Kit *et al.*, 1963). The cultures were grown in a 1:1 mixture of Eagle's medium, (H-MEMd) supplemented with nonessential aminoacids, 3 mM L-glutamine and 1 mM pyruvate sodium, and RPMI-1640; the mixture was further supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml) and glucose (2.5 mg/ml). All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were passaged every 2-3 days. Attached cells were disaggregated with phosphate-buffered saline (0.15 M NaCl/0.01 M sodium phosphate, pH 7.2) supplemented with trypsin (0.5 mg/ml) and 0.02% EDTA. To form aggregates the cells were plated in bacteriological-grade Petri dishes onto a layer of 1.5% Bacto-agar (Difco Laboratories, Detroit, MN, USA) in complete medium. Differentiation was induced by incubating the cells for 4 days in the presence of 0.5 μM retinoic acid (Sigma). The aggregates were transferred to tissue culture dishes and incubated for 3 more days under tissue culture conditions without retinoic acid. A stock solution of retinoic acid (10^{-2} M) was prepared as described (Dráber and Stanley, 1984).

Selection of adhesion-defective mutants

Mutant cell lines were isolated by a method described by Grover *et al.* (1987). Briefly, P19X1 cells (0.1 x 10^6/ml) were treated either with EMS (0.2 mg/ml or 0.1 mg/ml) for 18 h or with MNNG (1μg/ml or 2 μg/ml) for 2 h. After washing in culture medium mutagen-treated cells were grown for seven days to allow mutant phenotype to become expressed. At the end of the expression period the cells (adherent as well as those floating in the culture supernatant) were trypsinized and plated on Petri dishes at a concentration of 0.6 x 10^6 cells/ml. After an overnight incubation the cell culture supernatant with weakly adherent and non-adherent cells was harvested by pipetting, the cells were washed and transferred to a new dish. The same procedure was repeated every third day for two months. After the last transfer the cells were cloned by limiting dilutions in 96-well plates. Isolated single cells were recloned.

Monoclonal antibodies

The hybridoma cell line producing TEC-01 antibody has been described (Dráber and Pokorná, 1984). This IgM-class antibody recognizes the same oligosaccharide sequence Gal(ß1→4) [Fuc(α1→3)]GlcNac as the anti-SSEA-1 antibody (Gooi *et al.*, 1981). Rat mAb TEC-04 of IgG class, which recognizes a protein epitope TEC-4 was isolated in this laboratory (Dráber *et al.*, 1989b). A monoclonal anti-Thy-1.2 antibody of the IgG_3 subclass, clone 1aG4, was obtained as hybridoma supernatant or ascites fluid as described previously (Dráber *et al.*, 1980). A rat monoclonal antibody (DECMA-1) recognizing the murine cell adhesion molecule uvomorulin (UMt) was kindly provided by Dr. Rolf Kemler from the Max-Planck Institute of Immunology, Freiburg, Germany.

Radioantibody binding assay

Thirty-µg aliquotes of immunoglobulins were iodinated by the chloramine-T method as described (Stanley and Carver, 1977). The specific activity of the ^{125}I-labeled antibodies was 0.9-1.8 x 10^7 cpm/µg. ^{125}I-labeled antibodies (10^5 cpm/tube) were mixed with 2.5 x 10^5 cells in H-MEMd supplemented with 1% BSA in a final volume of 100 µl. After 1 h at 4°C the cells were separated from unbound antibodies by centrifugation at 1200 x g for 10 min at 4°C through a layer of 12% BSA in H-MEMd in polypropylene microtubes (Beckman). Thereafter the tubes were frozen, the tips cut and the radioactivity bound to the cell pellet and the supernatant was counted separately in a gamma counter. Binding was calculated as percentage of the total radioactivity bound to the pellet.

Cell adhesion assay

Monolayer cultures were treated with PBS supplemented with trypsin (0.5 mg/ml) and 0.02% EDTA. After 15 minutes at 37°C and centrifugation the cells were dispersed into single cell suspension by two to three passes through a 30-gauge needle in cell culture medium. Then 1-2 x 10^6 cells were passaged in 3 ml medium and incubated in an atmosphere of 5% CO_2 over a 24 h period to determine the number of non-adherent or weakly adherent cells using a hemocytometer. The percentage of total cells remaining as floating cells was used as a measure of the proportion of non-adhering cells.

Cell aggregation assay

Cells grown as semiconfluent monolayers were washed in cell culture medium and then treated with Ca^{2+} and Mg^{2+} free medium (CMF) containing 37 mM NaCl/5.4 mM KCl/0.34 mM Na_2HPO/5.4 mM KH_2PO_4/1 mM EDTA. The cells were centrifuged and resuspended either in CMF medium or in CMF medium supplemented with 2 mM $CaCl_2$. 1-2 x 10^6 cells in 1 ml were transferred into a tissue culture plastic dish and incubated at 37°C on a gyratory shaker (Elpan type 358S) at 80 rpm. The aggregation was stopped with a gentle swirling of the dish and subsequent addition of 0.5 ml 2.5% glutaraldehyde in PBS. In a preliminary experiment it was determined that this fixation procedure does not cause any artificial aggregation or dissociation of preformed aggregates. To measure cell aggregation, the total particle number in cell suspension was counted in a Coulter counter (model Z_B: Coulter Electronics, Hertfordshire, England) with a 100 µm aperture. Percentage was calculated as the percentage reduction in total particle number from time zero.

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