Vol. 106, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1982 Pages 236-242

"NOVEL CORRELATION BETWEEN GREATER CELL ADHESION TO SUBSTRATUM AND AN INCREASED ASSOCIATION OF CELL SURFACE PROTEINS WITH POLYPEPTIDES INVOLVED IN ACTIN POLYMERIZATION"

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Received April 5, 1982

SUMMARY. The fraction of actin-associated proteins that cosediments when cell extracts are lysed and incubated under conditions that promote in vitro actin polymerisation is enriched in actin and in a 190 kd protein when prepared from non-tumorigenic epithelial cells as compared with their less adherent, tumorigenic counterparts. Surface-iodinated proteins of about 145 kd and 190 kd cosediment with actin-associated proteins when the latter fractions are prepared from the non-tumorigenic cells and from the tumor cells which become more adherent after growth in bromodeoxyuridine. Surface proteins from poorly adherent tumor cells show much lower or negligible association with actin-associated proteins, and the absence of the prominent 190 kd polypeptide which cosediments with actin in extracts from non-tumorigenic, adherent cells. Our results suggest an important role for actin-associated proteins that participate in interactions with cell surface proteins, in events that influence cell adhesion to substratum.

INTRODUCTION. Recent reports from our laboratory have shown that phenotypically normal fibroblasts and epithelial cells which show increased adhesion to a growth substratum, reveal cathodic forms of surface-associated fibronectins (1), a result in agreement with other independent findings indicating that "normal" fibronectin is less phosphorylated than that found in transformed cells (2). It has also been observed that both "normal" and tumor epithelial cells can be made to increase their adhesion to a substratum by growth in the presence of iododeoxyuridine (IUDR) or bromodeoxyuridine in an effect which also correlates with increased levels of surface fibronectin (3) and the expression of a less negatively charged form of the same protein (1, 4). However, the molecular determinants of cell adhesion to substratum remain without complete elucidation. Our earlier experiments also revealed that the increased levels of cytoskeleton-associated fibronectin in tumor cells which show increased adherence after growth in IUDR, coincide with changes in other cytoskeleton-associated proteins, including actin (4). Other earlier immune fluorescence studies revealed a marked disorganization of

0006-291X/82/090236-07$01.00/0

actin-containing filamentous cables in tumor cells (5) and more recent electron microscopic data has revealed a reversion in the disorganization of the microfilament system in tumor cells grown with halogenated pyrimidines (6). Since surface-associated fibronectin and actin show coincident expression in adherent fibroblasts as revealed by immune fluorescence (7) and electron microscopy (8) we have now investigated the possible differential association of preiodinated-surface proteins and the protein fraction that results when actin is allowed to polymerise in vitro (9) using adherent epithelial cells, their tumorigenic poorly adherent counterparts and the latter cells in which adherence to substratum is increased by growth with halogenated pyrimidines (1, 3). The results to be presented show a novel defective interaction of surface polypeptides and actin-associated proteins in tumor cells, which correlates with low adhesion to substratum.

MATERIALS AND METHODS

Cell Cultures. WIRL-3C is an untransformed epithelial cell line which does not survive in the aggregate form above an agar base that prevents cell attachment, does not plate in soft agar, and does not form tumors in nude mice. R72/3 is an epithelial cell line derived from a tumor produced by a spontaneously transformed WIRL-3 subline. It grows well in the aggregate from above an agar base, revealing a 37% plating efficiency in soft agar and the ability to form tumors in mice (10). Cells were seeded to give subconfluent cultures after 72 hours of growth in Roswell Park Memorial Institute Tissue Culture Medium 1640, supplemented with 10% fetal calf serum, including 2.5 µg/ml bromodeoxyuridine (BrdU) whenever indicated. After growth in bromodeoxyuridine, R72/3 cells failed to grow is soft agar, resembling their parent untransformed WIRL-3C cells (3). Also after growth with halogenated pyrimidines, both cells have revealed increased adherence to substratum as revealed by significant resistance to detachment following exposure to 0.5 mM EDTA or 1 M urea in isotonic saline (1, 4).

Enzymatic Radioiodination. This was carried out using 40 µg lactoperoxidase (14.7 IU/mg; Calbiochem, San Diego, Calif.), 5 µg glucose oxidase (110 IU/mg; Worthington Biochemical Corp., Freehold, N.J.)., and 1.5 mCi $Na^{125}I$ (NEZ-033H; New England Nuclear, Boston, Mass.), in phosphate-buffered saline consisting of 0.9% NaCl in 0.115% Na_2HPO_4 and 2% KH_2PO_4 (pH 7.2) with 5 mM glucose, using 1.8×10^6 cells per iodination for 10 min at 22° as described elsewhere (1).

Assay of Interaction between Surface Proteins and Actin-Associated Proteins. This was carried out using surface iodinated cell monolayers labelled as described above. Cells were detached by exposing monolayers to 0.5 mM EGTA in 0.15 M NaCl, followed by cell lysis in sucrose 0.34 M, ATP 1 mM, dithiothreitol 1 mM, Tris-maleate 40 mM pH 7.9, EGTA 1mM; 0.5% Triton-X-100, phenyl methyl sulfonyl fluoride (PMSF) 0.1 mM. Extracts were centrifuged at 100.000 X g for 1 hour at 4° to yield a pellet (P_1) and a supernatant which was made 5 mM in Mg^{2+} and 0.1 M KCl to promote in vitro actin polymerisation during a 60 min incubation at 25°C (9). The iodinated components that became associated with polymeric actin and actin-associated proteins (AAP) were collected by centrifugation at 15.000 X g for 30 min, yielding also a soluble fraction (S). All fractions were exposed to a 5 X dissociating mixture to give a final concentration of 2% SDS, 0.1% β-mercapthoethanol, 0.1 M Tris pH 6.8, 2 mM PMSF and heated at 90° for 3 min for subsequent electrophoretic analysis in 7.5% SDS-polyacrylamide gels (1).

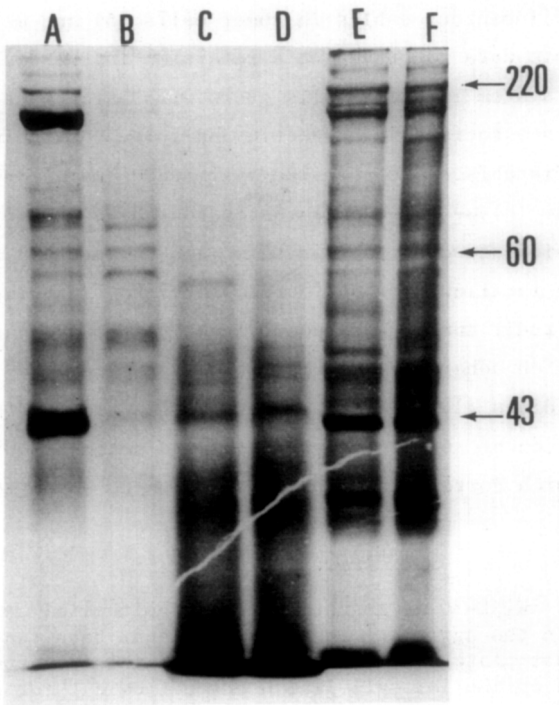

Fig. 1 Effect of Cell Transformation on the Polypeptide Composition of Fractions resulting from conditions that permit in vitro Polymerization of Actin.

Non-tumorigenic WIRL epithelial cells and their tumorigenic counterparts R72/3 cells were grown and lysed as described under Materials and Methods to prepare P_1, AAP and S fractions for electrophoretic analysis in 7.5% gels (1).

A, C and E correspond to AAP, S and P_1 fractions from WIRL cells.
B, D and F correspond to AAP, S and P_1 fractions from R72/3 cells.

RESULTS

The composition of the actin-associated protein fraction that copolymerised with actin in vitro in the presence of KCl and Mg^{2+} and the other soluble and sedimentable fractions that resulted from the same experiment are presented in Fig. 1. The AAP fraction from phenotypically normal epithelial cells revealed the presence of several components of similar migration to those seen in the same fraction prepared from the corresponding transformed counterparts cells, with the exception of a highly prominent 43 kd actin, an equally prominent 190 kd protein and a less prominent 220 kd polypeptide, which nevertheless was less obvious in the transformed cell fraction (Fig. 1A, B).

The soluble fraction remaining after low speed centrifugation of the AAP fraction was found to be similar in both normal and transformed cells with

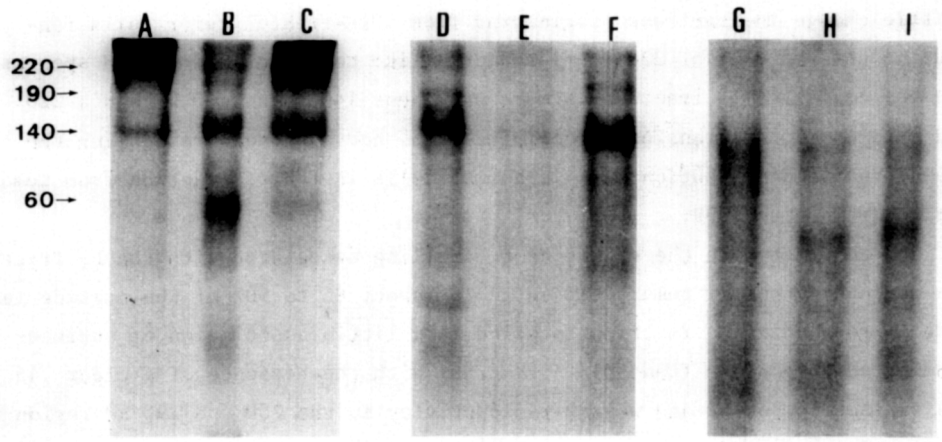

Fig. 2 Association of Cell Surface Proteins with the AAP fraction involved in Actin Polymerization.

Surface-iodinated monolayers from WIRL, R72/3 and R72/3 cells grown for 72 hours with bromodeoxyuridine, were detached and lysed under conditions described in Materials and Methods to assay interaction of surface proteins and actin-associated proteins.

A, D, G, correspond to iodination label associated with the P_1, P_2 and S fractions from WIRL cells.
B, E, H, correspond to iodination label associated with the P_1, P_2 and S fractions from R72/3 cells.
C, F, I, correspond to iodination label associated with the P_1, P_2 and S fractions from R72/3 cells grown with bromodeoxyuridine.

the exception of a protein component migrating in the 60 kd region only in the S fraction from normal cells (lanes C, D). Comparison of the protein composition of the sedimentable fractions P_1 obtained at high speed centrifugation revealed a similar but not identical pattern in normal and transformed cell fractions (lanes E, F).

The presence of similar proportions of 220, 170 and 43 kd protein components in the P_1 fractions from normal and transformed cells but not in the AAP fraction resulting from conditions that promote *in vitro* actin polymerisation argued in favour of a transformation-associated defect in actin-associated proteins. Similar results to those shown in Fig. 1 were observed when the same experiments were carried out with cells labelled with ^{3}H-aminoacid mixture (not shown). We then thought it worthwhile to investigate whether epithelial cell surface proteins were able to interact *in vitro* with AAP components and whether this interaction differed in normal and tumor liver epithelial cells, and in the latter cells in which adherence and modified expression of cytoskeleton associated actin and fibronectin are affected by growth with halogenated pyrimidines (1, 3, 4).

Electrophoretic analysis of the distribution of surface iodinated extracts in the P_1, P_2 and S fractions is presented in Fig. 2. The P_1 fraction revealed a similar pattern in extracts from normal and tumor cells, with

little change in fractions originating from IUDR-treated tumor cells featuring the presence of 220 kd fibronectin like component and 140 kd species in the normal cells fraction, a more prominent 140 kd and 60 kd and a 220 kd species in the transformed cell fraction, and a pattern resembling the quantitative distribution seen in normal cells in the P_1 fraction from tumor cells grown with IUDR.

In contrast with the significant labelling associated with the P_1 fraction in iodinated control tumor cell extracts (about 85 to 90% of the surface label), the corresponding P_2 fraction revealed very little association of surface-iodinated components (Lane E) in contrast with the presence of a clear 145 kd surface component and weaker radioactivity in the 200 and 190 kd region in the corresponding fractions from IUDR-treated tumor cells and normal cells. Very little radioactivity corresponding to about 5-7% of the surface label was detected in the soluble fractions, which revealed weak labelling in the 140 kd and 60 kd region, in tumor cell fractions and labelling mostly in the 140 kd in the corresponding fractions from phenotypically normal cells.

The yield of AAP and the association of the 145 kd surface protein with AAP observed in normal and IUDR-grown tumor cells was markedly decreased when the Mg^{2+}/KCl mediated polymerisation of actin and AAP was assayed at 4°instead of 25°, in agreement with others (9).

A similar inhibition of the association of the 145 kd with AAP was observed when such interaction was allowed to take place in the presence of 5 μg/ml cytoschalasin B known inhibitor of actin polymerisation (11).

Since our results suggested that the expression of the transformed phenotype altered significantly the composition of AAP which cosediment with actin under conditions that permit *in vitro* actin polymerisation in normal epithelial cells and since the defective association of surface labelled proteins with AAP could be counteracted in P_2 fractions from tumor cells grown in IUDR, we tested whether some of the polypeptide changes associated with the expression of transformation phenotype in the P_1, P_2 and S fraction were also counteracted by growth in IUDR. However, the corresponding protein gels revealed that IUDR-grown tumor cells yielded S and P_2 fractions similar to those shown in Fig. 1 for control tumor cells (not shown). Such observations suggest that the defective association of surface associated proteins with AAP seen in control tumor cells which appears to be partly corrected after growing them with IUDR cannot be correlated with any obvious acquisition or loss of polypeptides from the AAP fraction. Instead our data that imply the association of surface-proteins and AAP components correlate better with a restricted expression of the transformed phenotype which includes the increased cell adherence seen in normal and IUDR-grown tumor cells, both in previous studies (1, 4) and in the present study (not shown).

DISCUSSION

We have now shown that surface labelling of adherent cells, and analysis of the distribution of the surface label in extracts prepared under conditions that permit *in vitro* actin polymerisation, can be used as a novel assay to detect differences between phenotypically normal adherent epithelial cells, their poorly adherent tumorigenic counterparts and the corresponding tumor cells in which adherence is increased by growth in the presence of halogenated pyrimidines like bromo and iododeoxyuridine (1, 3, 4). The latter results suggest that halogenated pyrimidines may affect cell adherence to substratum by influencing the interaction of external proteins and polypeptides involved in actin polymerisation. Our data showing the *in vitro* association of AAP and surface proteins, may be related to the transmembrane association implied from ultrastructural studies of adherence-related fibronectin and actin filaments (8). However, in agreement with other findings indicating that most of the fibronectin remains associated with the Triton-insoluble cytoskeletal matrix (12), our data also show that most of the fibronectin remains in the Triton-insoluble P_1 fraction that sediments at 4°after 1 hour centrifugation at 100.000 X g. The most important association with AAP is observed in the Triton-soluble P_2 fraction which revealed a 145 kd surface component and the less prominent association of external proteins of about 200 and 190 kd cosedimenting with polymeric actin.

Although we cannot exclude at present that the 200 kd and 190 kd surface components that cosediment with the AAP P_2 fraction correspond to partly cleaved fibronectin fragments, nevertheless the proportion of such components to the external 145 kd protein in the AAP fraction is low, suggesting that the latter 145 kd component may be more important in the interaction of cell surface proteins with AAP and polymeric actin in the present system, or that a greater interaction of fibronectin with AAP is obscured because of the marked association of undergraded fibronectin with the Triton-insoluble matrix (12).

Nevertheless, our data clearly emphasize that in addition to the involvement of fibronectin in cell adherence, other surface components like the 145 kd protein that associates with AAP may be relevant in substituting for or cooperating with fibronectin in mediating cell adherence.

In addition to showing this novel correlation between increased adherence to substratum and the association of 145 kd surface components with actin-associated proteins, an additional novel finding is the observation that the fraction that cosediments with polymeric actin is enriched in a 190 kd protein which accompanies an equally prominent actin only in phenotypically normal epithelial cells which are more adherent and do not form tumors in nude mice.

Since epithelial cells of differing tumor ability are difficult to distinguish on the basis of morphological and biochemical criteria, the present results showing that the composition of actin-associated proteins and the interaction of surface proteins is modified in poorly adherent tumorigenic epithelial cells, suggest an important role for such proteins in cell adherence and epithelial cell transformation.

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