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LOSS OF FIBRONECTIN AMONG THE SELECTIVE SURFACE PROTEIN CHANGES ASSOCIATED WITH p-FORMALDEHYDE PROMOTED MEMBRANE VESICULATION

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SUMMARY p-formaldehyde-promoted membrane vesiculation of preiodinated cultures lead to the release of surface proteins of about 68 kd in normal and transformed rat liver epithelial cells and rat fibroblasts, concurrent with a marked decrease in surface-associated fibronectin. Membrane vesiculation in the presence of soybean trypsin inhibitor permitted the detection of an 18 kd surface protein in membrane vesicles and the increased expression of a 70 kd external component in normal but not in transformed epithelial cells.

Our results show that the membrane vesiculation process is associated both with the release and selective degradation of specific cell surface proteins in a process which may involve surface protease activation. Our data also suggest the potential of the chemical vesiculation process as a probe to monitor differences in surface topography between different cell types.

INTRODUCTION. It has been shown that normal and transformed mouse embryo cells and a variety of other monolayer cell lines can be induced to shed plasma membrane vesicles by exposure to p-formaldehyde and dithiothreitol. However, such studies concentrated mostly on the characterization of the vesicles released, without identifying the cell surface protein changes occurring in the cells exposed to the vesiculation process (1-2).

Recent studies from our laboratory have indicated that the chemical vesiculation process can be used to reveal a differential susceptibility of cell surface proteins in melanoma variants, with differing metastatic potential (3). Other studies using fluoresceinated gelatin have indicated that surface fibronectin that usually binds to gelatin, is virtually eliminated when cells are preexposed to p-formaldehyde for 30 min (4).

Since all the above studies suggest the potential of using the chemical vesiculation process to probe biochemical differences in cell surface topography, we have now analyzed the fate of fibronectin and other surface iodinated proteins from normal and transformed cultures following their exposure to the chemical vesiculation process. In this report, we demonstrate that p-formaldehyde vesiculation is a useful probe to monitor the dynamics of cell surface proteins.

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MATERIALS AND METHODS

Cell and Cell Cultures. a) WIRL-3C is an untransformed epithelial cell line which does not survive in the aggregate from 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 form above an agar base, revealing a 37% plating efficiency in soft agar and the ability to form tumors in mice (5,6). b) NKR cells are normal rat kidney fibroblasts that grow forming flat, highly adherent contact-inhibited monolayers, which have been shown to contain surface-associated fibronectin (7). c) Rat-1 normal fibroblasts have low levels of fibronectin (4) and also form highly contact-inhibited flat monolayers (4).

All cultures were grown to subconfluency in RPMI 1640 medium supplemented with 10% fetal calf serum.

Surface radioiodination. This was carried out using 40 µg lactoperoxidase (14.7 I.U/mg; Calbiochem, San Diego, Calif.), 5 µg glucose oxidase (110 I.U/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 0.2% KH_2PO_4 (pH 7.2) with 5 mM glucose, using $1.8x10^6$ cells per iodination for 10 min at 22°. Whenever indicated metabolic labelling was carried out with 100 µc ^{35}S-methionine (NEG 009H; NEN) using also 1.8×10^6 cells per 9 cm dish. Electrophoretic analysis of the cell extracts was carried out as described elsewhere (6) in 7.5% SDS-polyacrylamide Laemmli gels (8) followed by autoradiography of the vacuum-dried gels (6).

Chemical Vesiculation of cells. This was carried out by exposing preiodinated cell monolayers to 25 mM p-formaldehyde (pCH_2O) and 2 mM dithiothreitol (DTT) in phosphate-buffered saline containing 0.75 mM $CaCl_2$ and 0.5 mM $MgCl_2$ for 60 min at 37° (1-3). Shed vesicles were collected after removal of possible aggregates at 1.500 X g for 10 min and subsequent harvesting at 16.000 X g for 20 min. Whenever indicated, soybean trypsin inhibitor (STI, Sigma Cat N° 7-9003) was added during vesiculation at a final concentration of 10 µg/ml, after iodination of cells and exhaustive removal of the iodinating reagents.

RESULTS

Surface iodination of "normal" WIRL epithelial cells revealed a major 220 kd fibronectin-like component which disappeared when cells were exposed to p-formaldehyde-promoted vesiculation (Fig. 1). Although this loss occurred even in the presence of soybean trypsin inhibitor, the latter permitted the increased expression of a surface-associated 68 kd protein and the recovery of an 18 kd and 68 kd species in the vesicle fraction shed from the preiodinated cells (Fig. 1, lanes C, E). An identical experiment with the corresponding "transformed" R72/3 cultures derived from the WIRL cells showed the presence of a 140 kd species and a weaker but detectable 220 kd component (Fig. 2). Chemical vesiculation of such cells also led to the preferential disappearance of the surface-associated 220 kd species and to the detection of label at the top of the gel with no expression of a surface-associated 70 kd species, comparable to that seen in similarly treated normal epithelial cells (see Fig. 1C, 2B). However,

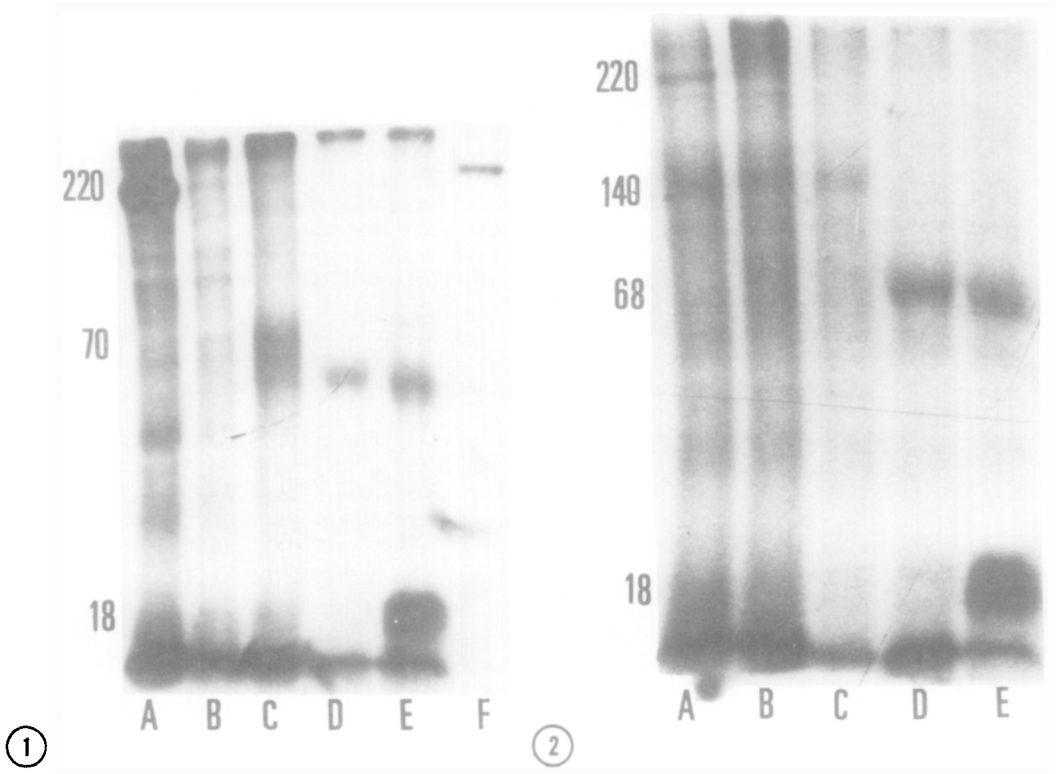


Figure 1. Influence of Soybean Trypsin Inhibitor on the Cell Surface Protein Changes Promoted by Chemical Vesiculation in Normal Epithelial Cells.
Surface-iodinated WIRL Cultures were subjected to pCH_2O/DTT vesiculation, and subsequently analyzed by electrophoresis and autoradiography, as described under Materials and Methods.
A, surface-associated label from control cells
B, surface-associated label from cells exposed to vesiculating conditions
C, same as B, but with vesiculation carried out in the presence of STI
D, shed vesicles form B
E, shed vesicles from C
F, aliquot from A reacted with anti-fibronectin serum.

Figure 2. Cell Surface Protein Alterations Associated with Membrane Vesiculation in Transformed Epithelial Cells.
Surface-iodinated R72/3 cells were labelled and analyzed as indicated in Fig. 1.
A, surface-associated label from control cells
B, surface-associated label from cells exposed to vesiculating conditions in the presence of STI
C, same as B, but without STI
D, shed vesicles from C
E, shed vesicles from B.

the vesicle fraction from transformed cells also revealed the presence of a 68 and 18 kd proteins when vesiculation was carried out in the presence of soy-bean trypsin inhibitor (Fig. 2).

An additional experiment with the WIRL and R72/3 epithelial cultures was carried out by metabolic labelling of the cells with ^{35}S-methionine in order to determine whether the decrease in surface-

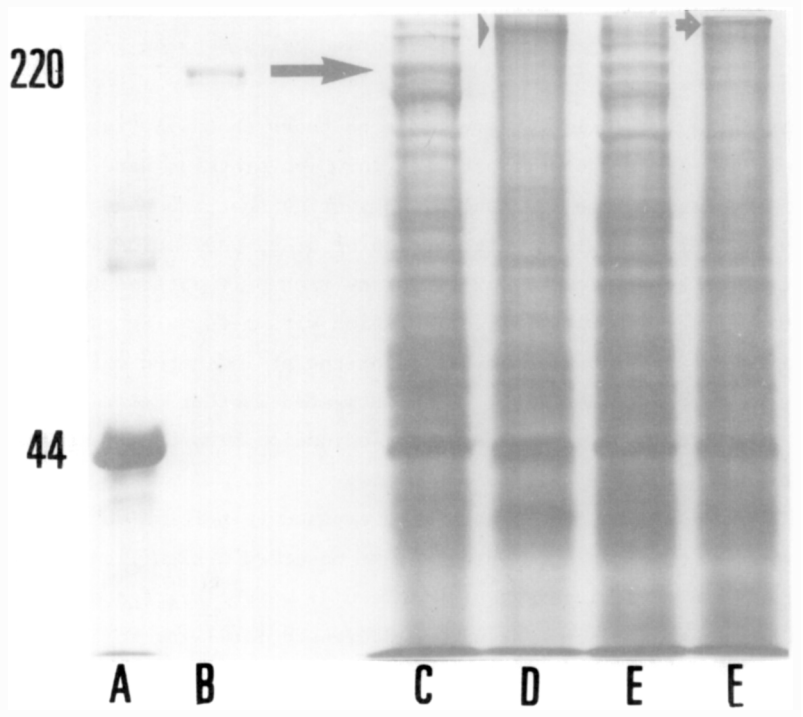


Figure 3. Polypeptide Changes Associated with Membrane Vesiculation of Normal and Transformed Epithelial Cells.
Normal WIRL cells and their transformed counterparts R73/3 cells were grown for 36 hours in complete medium supplemented with ^{35}S-methionine. Subsequently cultures were washed to remove unincorporated isotope and subjected to vesiculation whenever indicated, followed by lysis and electrophoretic analysis as described in (6) and in Materials and Methods. Aliquots of the same samples were also subjected to electrophoretic examination and subsequent 0.05% Coomassie blue staining, with essentially similar results.
A, actin marker
B, fibronectin marker
C, extract from control normal WIRL cells
D, same as C, but from cells exposed for 1 hour to p-formaldehyde /DTT
E, extract from control transformed R72/3 cells
F, same as in E, but from cells exposed for 1 hour to p-formaldehyde /DTT
The big arrow shows the position of fibronectin in C and E, the arrow head indicates the slow-moving polypeptide increased in D and the small arrow shows the slower-migrating polypeptide increased in F.

associated-fibronectin also involved a loss in total cell fibronectin. A comparison of gel patterns from extracts of control cells and those exposed to the vesiculation conditions also revealed that the latter lacked several components showing the absence of a 220 kd species which comigrated with reference fibronectin in control cell extracts.

In agreement with our observations from Fig. 1 and 2 suggesting that the vesiculation process involved similar but not identical changes in extracts from normal and transformed cells, we also observed the presence of a high molecular weight polypeptide in extracts from

normal cells and the expression of a protein of an apparently larger size in extracts from transformed cells exposed to the vesiculation process (Fig. 3).

Essentially similar observations to those shown in Fig. 3 were observed in electrophoretic patterns in which proteins were stained by 0.05% Coomassie blue (not shown) suggesting that the changes reported above in cells labelled with methionine or by iodination are not restricted to methionine -or tyrosine- rich polypeptides (not shown).

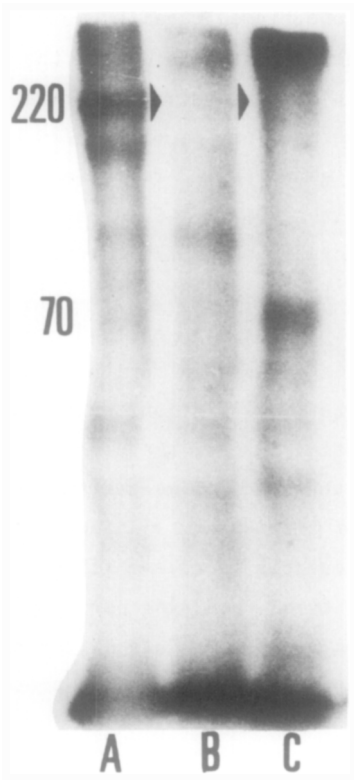
Since the conditions of vesiculation with p-formaldehyde usually implied a 60 to 120 min incubation of the preiodinated cells subsequent to their labelling, we investigated whether cells exposed for 10 min to p-formaldehyde prior iodination also underwent cell surface change.

When normal NRK fibroblasts were exposed to p-formaldehyde for 10 min and then surface-iodinated, we observed a significant accumulation of radioactivity in a high molecular weight aggregate and in a 70 kd component concurrent with a decreased labelling in the 220 kd fibronectin region. A similar decrease in the expression of the 220 kd species was observed in preiodinated NRK cells after exposure to vesiculation conditions (Fig. 4).

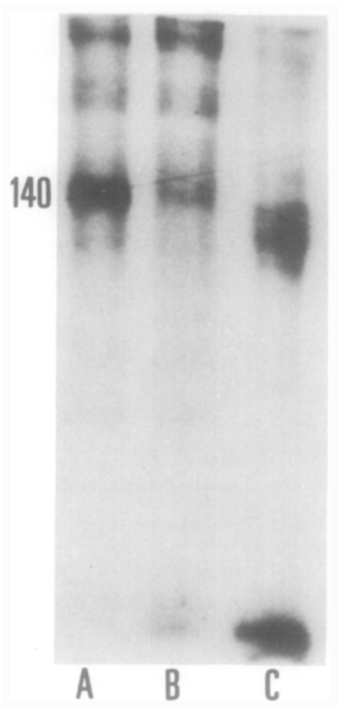
Since normal rat-1 cells have been shown to posses lower levels of fibronectin-like gelatin binding surface proteins (4), we tested the effect of chemical vesiculation on such cells. In contrast with the marked decrease in the 220 kd observed with WIRL and NRK normal cells rat-1 cells revealed a marked decrease in a surface-associated 140 kd species concurrent with the expression of a 130 kd component in the vesicle fraction (Fig. 5).

DISCUSSION

We have now shown that the membrane vesiculation process mediated by the p-formaldehyde DTT treatment may involve a novel protease activity. This is suggested by our data revealing the preferential loss of surface-associated fibronectin from fibronectin-containing cells, (6, 7), and by the cleavage of a surface-associated 140 kd protein from rat-1 cells, which have low levels of fibronectin (4), after the chemical vesiculation process. Additional support for the involvement of a surface-associated protease in the chemical vesiculation process comes from the experiments in which soybean trypsin inhibitor is included during vesiculation. Under such conditions, we were able to detect in the shed vesicle fraction the presence of an 18 kd iodinated protein, which resembles a similar component found following vesiculation of poorly metastatic melanoma (3). An observation which argues



(4)



(5)

Figure 4. Effect of p-formaldehyde /DTT treatment on the Surface Proteins of Normal Fibroblasts.
Surface-iodinated NRK cells were iodinated either prior to or after exposure to pCH_2O/DTT and analized as indicated in Fig. 1.
A, surface-associated label from iodinated control cells.
B, surface-associated label from iodinated cells exposed to vesiculating conditions for 60 min at 37°
C, surface-associated label from cells exposed to pCH_2O/DTT for 10 min at 37° and then iodinated.

Figure 5. Fibronectin-independent Alteration of Cell Surface Proteins Associated with the Vesiculation Process
Rat-1 cells were iodinated and analyzed as described under Materials and Methods in the Legend to Fig. 1.
A, surface-associated label from control cells
B, surface-associated label from cells exposed to vesiculating conditions
C, shed vesicles from B.

against a non-specific random protease activity in cells exposed to vesiculating conditions comes from the electrophoretic protein patterns of normal and transformed epithelial cells. Such patterns both show an increase in slow-migrating polypeptides apparently larger than fibronectin revealing also an interesting unequal effect on normal and transformed cells.

The experiments in which surface-iodinated cultures were exposed to the vesiculating process involved parallel assays with antifibronectin

serum to detect whether the 220 kd fibronectin detectable in control cultures appeared as a smaller component still recognised by the specific antiserum. However, immune precipitation analysis carried out as described previously (6) revealed the presence of only a 220 kd component in a reaction antagonized by 20 μg unlabelled refference fibronectin only in control iodinated cells (not shown). Such results appear in essential agreement with other findings in which fibronectin is completely undetectable after formaldehyde treatment the cells (4).

In spite of the well known role of fibronectin in cell adherence (see 9, 10 for recent reviews) it is of interest WIRL epithelial cells and NRK fibroblasts, remain attached after vesiculation, which involves the loss of surface-associated fibronectin. Hence, the present observations permit an additional approach to the study of mechanisms involving the fibronectin-independent adherence of fibroblasts (11) and also permit the study of enzymes which may preferentially cleave fibronectin (12).

Finally, in addition to the possibility of obtaining partly purified cell surface proteins after vesiculation (1-3), our data offer the potential of obtaining cells devoid of specific cell surface proteins for either biochemical or immunological experiments.

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