Effect of Malignant Transformation and Arginine Limitation on Fibronectin and Other Cell Surface Macromolecules of Liver Epithelial Cultures

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ABSTRACT

Surface labeling of "normal" nontumorigenic rat liver epithelial cells permitted the detection of a 140,000-dalton species and of another 230,000-dalton molecule that comigrated with fibronectin, an external transformation-sensitive protein of fibroblasts. Reaction of surface-labeled normal liver epithelial cells with anti-fibronectin serum led to the selective recognition of the 230,000-dalton component, which revealed a peptide composition similar to that of the homologous fibroblast fibronectin. Similar experimental conditions with the tumorigenic liver epithelial cell counterpart revealed decreased labeling in the fibronectin region and preferential labeling in the 140,000- and 60,000-dalton regions. Arginine limitation was found to produce growth arrest in both normal and malignant liver epithelial cells, concurrent with a marked decrease in the labeling of the surface-associated fibronectin and different surface macromolecular alterations in normal and transformed cells. Mild proteolysis combined with brief neuraminidase treatment did not produce marked alterations in tumorigenic cell surface labeling but did lead to a decrease in the fibronectin of normal cells and to the increased expression of other transformation-associated changes in surface macromolecules different from fibronectin.

Our results show the involvement of an arginine-regulated fibronectin and other cell surface macromolecules among the changes that occur during the conversion of normal liver epithelial cells to a malignant state.

INTRODUCTION

Although many important advances related to in vitro transformation and carcinogenesis have been carried out with fibroblasts in tissue culture, conditions that limit the multiplication of epithelial cells appear to differ from those of fibroblasts (6, 7, 9).

A significant advance in the definition of molecular differences in cell surface components of normal and malignant cells took place with the discovery of the LETS3 glycoprotein or fibronectin, which is known to be absent or decreased in most transformed fibroblasts but present in the corresponding normal cells (for reviews, see Refs. 10, 20, and 22).

Recently, studies on fucose-labeled components, released by proteolysis, have shown that both transformed epithelial cells and fibroblasts exhibit a group of glycopeptides which elute earlier on Sephadex G-50 than those obtained from comparable normal cells (16).

However, in addition to the reported absence of the LETS protein from the surface of rat hepatocytes (12), other recent immunofluorescence studies have revealed significant differences between confluent fibroblasts that build a massive network of fibrillar LETS protein and confluent epithelial cells which are unable to do so (3).

Due to the particular importance of epithelial cells in the origin of carcinomas, and because of the lack of information to date on the surface macromolecules that reflect transformation-associated changes of epithelial cells, we decided to investigate possible differences in the surface labeling of untransformed, nontumorigenic Wistar rat liver epithelial cells with those of their transformed, tumorigenic counterparts, under conditions in which proliferation is allowed or prevented in both cell types.

Since a low serum concentration does not inhibit the growth of malignant cells as effectively as it does with normal cells (14), we have now used an arginine-deficient medium (1) to obtain nonproliferative cell populations of the normal and tumorigenic cells.

The results to be shown reveal the involvement of fibronectin and other novel surface macromolecules among the transformation-association changes affecting liver epithelial cells.

MATERIALS AND METHODS

Cells and 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 form above an agar base, revealing a 37% plating efficiency in soft agar and the ability to form tumors in mice. Both cells were recently characterized (5, 19) and kindly given to us by Dr. Robert C. Ting (Biotech Research Laboratories, Rockville, Md. 20852).

Rat liver fibroblasts show density-dependent inhibition of growth and the presence of LETS protein on their surface (see "Introduction").

Cultures were maintained in Roswell Park Memorial Institute Tissue Culture Medium 1640, supplemented with 10% fetal calf serum, unless otherwise indicated. Experiments involving arginine limitation were carried out by exposing cells to an arginine-depleted medium supplemented with dialyzed fetal calf serum for 18 hr (1).

Enzymatic Radioliodination. This was carried out using 40 μg lactoperoxidase (14.7 IU/mg; Calbiochem, San Diego, Calif.), 5 μg glucose oxidase (110 IU/mg; Worthington Bio-
chemical Corp., Freehold, N. J.), and 1.5 mCi Na\textsuperscript{125I} (NEZ-033H; New England Nuclear, Boston, Mass.), in phosphate-buffered saline consisting of 0.9% NaCl in 0.115% Na\textsubscript{2}HPO\textsubscript{4} and 0.2% KH\textsubscript{2}PO\textsubscript{4} (pH 7.2) with 5 mM glucose, using 1.8 x 10\textsuperscript{6} cells per iodination for 10 min at 22° as described elsewhere (18).

**Immunoprecipitation with Anti-Fibronectin Serum and Analysis of Extracts for Electrophoresis and Autoradiography.** The antiserum used in the present study was prepared against purified fibronectin from normal baby hamster cells and was kindly supplied by Dr. R. C. Hughes (National Institute for Medical Research, Mill Hill, London, England). The antiserum was prepared by purification of the 230,000-dalton fibronectin-containing fraction with gelatin-containing Sepharose columns (8, 15). The antiserum was found to give a single precipitation line against similarly prepared fibronectin from normal rat kidney cells. Immunofluorescence analysis showed reactivity in normal rat and mouse fibroblasts but not with the corresponding transformed fibroblasts. Extracts from 1.8 x 10\textsuperscript{6} cells were prepared by freezing and thawing 3 times in Buffer A (0.15 ml), consisting of 5 M urea, 1% Triton X-100, 0.001 M phenylmethylsulfonyl fluoride, and 0.05 M Tris-HCl (pH 7.5). After removal of insoluble aggregates, the supernatants (0.12 ml) were preabsorbed with 0.02 ml of preimmune rabbit serum for 1 hr at 37° and a further 16 hr at 4°. After centrifugation at 12,500 X g for 20 min, the supernatants were reacted with 0.02 ml rabbit anti-fibronectin serum for collection, as described for the preimmunoprecipitates. Whenever indicated, peptide mapping by limited proteolysis was carried out by an adaptation of the procedure of Cleveland et al. (3) with resuspension in Buffer A. The immunoprecipitates were obtained by reacting surface-labeled cell extracts with anti-fibronectin serum, followed by a 30-min exposure at 37° to 20 µg Staphylococcus aureus protease V8 and subsequent electrophoresis. Samples for electrophoretic analysis were made 2% in sodium dodecyl sulfate, 0.002 M phenylmethylsulfonyl fluoride, 0.1 M β-mercaptoethanol, and 0.1 M Tris-HCl (pH 6.8) and heated for 3 min to 90° (11). Electrophoresis was carried out in 3 to 15% gradient gels for surface-labeled cells and in 5 to 15% gradient gels for protease-treated extracts, as described by Laemmli (11), followed by autoradiography for the iodinated gels (18). Approximate molecular weights were estimated by the use of known protein standards (purified hamster fibronectin, phosphofructokinase A, albumin, myoglobin, and cytochrome c) exposed to parallel electrophoresis under identical conditions to those used for samples.

**RESULTS**

**Differential Effect of Arginine Limitation on the Surface Labeling of Normal and Malignant Liver Epithelial Cells.** Maintenance of normal and malignant liver epithelial cells in an arginine-deficient medium for 18 hr has been found to be suitable to obtain reversible nonproliferating populations of both normal and malignant cells (1). Hence, we investigated whether comparable conditions of arginine limitation which prevented cell doubling would lead to differential molecular alterations in surface macromolecules of normal and malignant epithelial cells.

For such a study, we used lactoperoxidase-catalyzed surface iodination of cultures to examine the effect of arginine depletion during an 18-hr period in medium otherwise supplemented with 10% dialyzed fetal calf serum. Comparative surface iodination was found to give significant exposure in the 140,000- and 60,000-dalton regions in control transformed epithelial cells (Fig. 1, Lane C) as compared with their normal counterparts (Fig. 1, Lane A), which showed greater labeling in the 230,000-dalton fibronectin-like region and in the 140,000-dalton region. Limitation of arginine was found to decrease the labeling in the fibronectin-like 230,000-dalton molecule of untransformed epithelial cells concurrent with an increased exposure in the 60,000-dalton region (Fig. 1, Lanes A and B). On the other hand, arginine limitation of the transformed epithelial counterparts led to an increased exposure in the 140,000-dalton region (Fig. 1, Lanes C and D), concurrent with an even more evident lower labeling in the 230,000-dalton region and increased labeling in previously unlabeled components of about 90,000 daltons (Fig. 1, Lane D).

In contrast with the epithelial cells tested, no comparable change was observed by surface labeling of rat liver fibroblasts similarly exposed to arginine limitation (Fig. 2), a result which may be due to a difference between cell types and is worth further investigation.

**Differential Effect of Arginine Limitation on the Metabolic Labeling of Normal and Malignant Liver Epithelial Cells.** To investigate whether the observed changes in the 230,000-dalton region in transformed and arginine-starved normal epithelial cells were due to decreased surface exposure and not to decreased synthesis, we carried out metabolic labeling of the corresponding cultures with \[^{3}H\]glucosamine. Labeling with \[^{3}H\]glucosamine showed decreased radioactivity in the 230,000-dalton region of the malignant cells, both in control cultures and in cells exposed to arginine limitation (Fig. 3, Lanes A and B). On the other hand, \[^{3}H\]glucosamine labeling of "normal" cultures in complete medium showed a band in the 230,000-dalton region in control cells (Fig. 3, Lane D), in contrast with the same cells limited in arginine which showed decreased levels of radioactivity in the 230,000-dalton region and an increased labeling in a glycosylated macromolecule of slower mobility than fibronectin (Fig. 3, Lane C).

**The 230,000-Dalton Transformation-sensitive Surface Component of Epithelial Cells is Antigenically Related to Fibronectin.** The results of the previous section showed that one of the clearer changes detected by surface labeling involved a transformation-sensitive component detected by methods similar to those that have led to the identification of fibronectin, the external transformation-sensitive protein of fibroblast (10, 20, 22). Hence, we thought it worthwhile to examine the antigenic relationship of epithelial and fibroblastic molecules of similar molecular weight and exposure. Fig. 4 shows that the 230,000-dalton external component detected by enzymatic iodination of epithelial cells (Lane A) can be selectively immunoprecipitated by anti-fibronectin serum (Lane B), which recognizes a macromolecule of identical size on the surface of normal fibroblasts (Lanes C, D, and E).

Control experiments in which anti-fibronectin serum was preabsorbed with purified immunogen markedly decreased the ability of the antiserum to recognize the 230,000-dalton macromolecule from epithelial cells (not shown).

To complement the data obtained by serological cross-reaction and coelectrophoresis of the epithelial 230,000-dalton surface molecule and the fibronectin, we carried out additional
comparison of the 2 macromolecules by peptide mapping following mild proteolysis (4).

A significant homology between the epithelial "fibronectin" and its fibroblastic counterpart was suggested by the similarity in the cleavage products obtained after proteolytic treatment of the rat liver epithelial and rat liver fibroblast surface macromolecules that were immunoprecipitated by anti-fibronectin serum (Fig. 5, Lanes B and C). In contrast, no comparable similarity was observed in an identical experiment using surface macromolecules reactive with anti-fibronectin serum from MRC-5 human fibroblasts (Fig. 5, Lane A).

The above results indicating limited homology between surface macromolecules of human and rat fibroblasts are in agreement with previous reports which have indicated pronounced differences in peptide maps of fibronectin-like proteins from several different species of fibroblasts (13).

Transformation-dependent Effect of Neuraminidase and Trypsin on Cell Surface Macromolecules of Liver Epithelial Cultures. Since a number of reports have shown different levels of sialic acid-containing glycopeptides in normal and malignant cells (21), we thought it worthwhile to investigate whether mild treatment with neuraminidase and trypsin prior to surface iodination would permit the detection of other transformation-associated changes in liver epithelial cells.

For this purpose, we treated normal and transformed epithelial cells with 0.1 μg of crystalline trypsin per ml and 1 unit of neuraminidase for 10 min at 37°C prior to iodination in order to study the possible labeling of surface proteins previously unexposed to enzymatic treatment.

In contrast to normal liver epithelial control cells, which showed most of their labeling in the 230,000-dalton region and little labeling in the 120,000-dalton region (Fig. 1, Lane A), the same cells treated with neuraminidase and trypsin prior to iodination revealed by gradient gel electrophoresis a significantly increased labeling in the 120,000-dalton region as well as in the 160,000-dalton region, concurrent with a detectable lower level of labeling in the 230,000-dalton region (Fig. 6, Lane C), as compared with normal epithelial cells exposed only to mild proteolysis (Fig. 6, Lane D), which showed a prominent 230,000-dalton component.

In contrast, neither the 230,000-dalton component nor the 160,000- or 120,000-dalton molecules were detected in tumorigenic epithelial cells, even after neuraminidase treatment and mild or simple proteolysis (Fig. 6, Lanes A and B). Such results suggest that neuraminidase treatment and mild proteolysis can be used to evidence hitherto unreported differences in surface macromolecules of normal and tumorigenic liver epithelial cells.

DISCUSSION

In an attempt to separate growth-dependent from transformation-associated differences in surface properties of epithelial cells, we have now studied the external macromolecules of proliferating and nonproliferating transformed and normal liver epithelial cells, taking advantage of the fact that arginine limitation prevents cell doubling in both normal and transformed cultures (1).

In addition to the clear demonstration of fibronectin in normal cells and, to a much lesser extent, in transformed liver epithelial cells, we have now shown that fibronectin appears to decrease in resting normal epithelial cells exposed to arginine limitation, an effect not seen in the same cells arrested by serum deprivation. The latter cultures reveal a surface labeling pattern essentially identical to that seen in the corresponding control cultures grown in 10% serum, as shown in Fig. 1, Lane A.

Our finding of a different effect of growth arrest by serum limitation and arginine deprivation on cell-associated epithelial fibronectin resembles flow microfluorometric results which indicated that Swiss 3T3 cells arrested by isoleucine deprivation appear to be in a different point of the cycle compared to the same cells arrested by low serum (23).

Another interesting aspect of our studies was the observation of transformation-associated surface macromolecules that were different from fibronectin, which can be differentially detected in normal and in tumorigenic liver epithelial cells by arginine limitation or following exposure to neuraminidase and mild proteolysis.

The preliminary comparative studies or arginine deprivation using rat liver cultures of epithelial and fibroblastic morphology have revealed a preferential susceptibility of the epithelial cultures to undergo cell surface alterations as a result of arginine limitation. It will be of interest to define in future work whether the differential effect of arginine now observed is a more general difference between cells of mesenchymal and parenchymal types.

Another potentially worthwhile area of investigation would be the study of the possible functions and properties of the novel transformation-associated surface molecules now described for epithelial cell cultures.

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ADDITION

Since the submission of this manuscript, we have learned that Aaldovlic and Diamond (2) found that tumorigenic R72/3 tumorigenic liver epithelial cells do not show fibronectin on their surface but reveal instead prominent surface radioactivity in the 130,000- and 60,000-dalton regions, in contrast to the WRL-3C normal liver epithelial cells which show preferential exposure of a 220,000-dalton molecule identified as fibronectin by immunofluorescence and its trypsin susceptibility. Such data are in essential agreement with our findings of surface components of about 130,000 and 60,000 daltons in tumorigenic R72/3 liver epithelial cells and with our description of an arginine-regulated transformation-sensitive epithelial 230,000-dalton protein, shown to be fibronectin by immunoprecipitation and comparative peptide mapping with homologous fibroblast fibronectin.

REFERENCES

Surface Markers of Malignant Epithelial Cells


Fig. 1. Differential effect of arginine limitation on the surface iodination pattern of untransformed and malignant epithelial cells. WIRL normal cells and R72/3 transformed cultures at an initial inoculum of about 1.5 x 10^6 cells/9-cm dish were grown for 20 hr in complete medium supplemented with 10% serum and subsequently exposed to a medium change for 18 hr under conditions of arginine depletion or addition in the presence of dialyzed 10% serum, as indicated in each case. Cells were then labeled by surface radioiodination for electrophoretic analysis, as described in “Materials and Methods.” A, WIRL cells iodinated after exposure to complete medium; B, WIRL cells iodinated after exposure to arginine-depleted medium; C, R72/3 cells iodinated after exposure to complete medium; D, R72/3 cells iodinated after exposure to arginine-depleted medium. Arrows, regions of more obvious changes after arginine limitation; numbers, daltons (in thousands).

Fig. 2. Surface iodination pattern of rat liver fibroblasts exposed to arginine limitation. Normal rat liver fibroblasts (1.5 x 10^6 cells/9-cm dish) were grown with complete medium and subsequently exposed to arginine depletion as described in the legend to Fig. 1. Cells were then labeled by surface radioiodination for electrophoretic analysis and immunoprecipitation with anti-fibronectin serum, as described in “Materials and Methods.” A, cells iodinated after exposure to complete medium; B, cells iodinated after exposure to arginine-depleted medium; C, immunoprecipitate from cells labeled as in B. Arrow, position of migration of the 230,000-dalton (230) fibronectin marker.
Fig. 3. Effect of arginine limitation on labeling of cellular glycoproteins of normal and transformed epithelial cells. Normal WIRL and transformed R72/3 cells were seeded at a concentration of 1.8 x 10⁶ cells/9-cm dish for 18 hr in medium supplemented with 10% serum and 50 µCi of [³H]glucosamine, whenever indicated. Cell lysis, electrophoretic analysis, and fluorography were then carried out as detailed previously (17). A, transformed R72/3 cells labeled during arginine limitation; B, transformed R72/3 cells labeled in complete medium; C, normal WIRL cells labeled in arginine-depleted medium; D, normal WIRL cells labeled in complete medium. Arrows, position of a 230,000-dalton (230) fibronectin standard. < >, position of a component increased in normal cells labeled in arginine-depleted medium.

Fig. 4. Antigenic relationship between fibroblastic fibronectin and surface macromolecules of epithelial cells. WIRL normal epithelial cells and NRK normal rat fibroblasts were seeded and grown in medium with 10% serum for 48 hr to give subconfluent cultures for surface labeling, immunoprecipitation with anti-fibronectin serum, and electrophoretic analysis, as described in "Materials and Methods" and in the legends to Figs. 1 and 2. A, WIRL rat epithelial cells; B, immunoprecipitate from WIRL cells; C, confluent NRK rat fibroblasts; D, subconfluent NRK rat fibroblasts; E, immunoprecipitate from cells labeled as in C. Arrow, position of migration of the 230,000-dalton fibronectin.
Fig. 5. Comparison of the homology of surface macromolecules antigenically related to fibronectin in fibroblasts and epithelial cells. Surface-iodinated monolayers (1.8 x 10⁶ cells) of WIRL rat liver epithelial cells, rat liver fibroblasts, and human MRC-5 fibroblasts were solubilized, preabsorbed, and then reacted with anti-fibronectin serum as described under "Materials and Methods." The immunoprecipitates were subsequently solubilized and exposed to limited proteolysis and electrophoretic analysis in 5 to 15% gradient gels, as described in "Materials and Methods." A, human MRC-5 fibroblasts. B, rat fibroblasts. C, rat WIRL epithelial cells.

Fig. 6. Effect of mild neuraminidase and protease treatment on the surface labeling of normal and tumorigenic liver epithelial cells. Replicate cultures of subconfluent monolayers of normal WIRL cells and tumorigenic R72/3 cultures (2 x 10⁶ cells/9-cm dish) were exhaustively washed to remove serum components and exposed to a 10-min treatment at 37°C with 0.1 μg crystalline trypsin per ml and 1 unit of neuraminidase (Sigma type IV) in medium without serum, whenever indicated. Subsequently, monolayers were washed with phosphate-buffered saline for iodination and electrophoretic analysis, as described in "Materials and Methods." A, R72/3 cells with neuraminidase and trypsin treatment prior to iodination; B, R72/3 cells with only trypsin treatment prior to iodination; C, WIRL cells with neuraminidase and trypsin treatment prior to iodination; D, WIRL cells with only trypsin treatment prior to iodination. Upper arrow, position of migration of a 230,000-dalton (230) fibronectin standard; Lower arrow, region of more obvious change in Sample C.
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