Vol. 99, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 31, 1981 Pages 414-421

# EFFECT OF CELL TRANSFORMATION AND IODODEOXYURIDINE ON THE CHARGE HETEROGENEITY OF LARGE EXTERNAL TRANSFORMATION-SENSITIVE PROTEINS

By

M. Rieber and M.S. Rieber

Centre of Microbiology and Cell Biology
Instituto Venezolano de Investigaciones Cientificas
Apartado 1827 Caracas 1010-A Venezuela

Received February 2, 1981

SUMMARY. Comparative two dimensional mapping of surface-iodinated WIRL epithelioid untransformed cells and their transformed counterparts, revealed in the latter a decreased labelling in the 220K region, particularly in cathodic components more evident in the untransformed cultures. A similar assay with phenotypically normal and morphologically transformed fibroblasts also revealed in the latter a preferentially decrease in cathodic 220K dalton species.

Growth of the normal epithelioid cultures in the presence of iododeoxyuridine correlated with both an increased cell adherence and with an enhanced cathodic migration of the 220K surface iodinated species cross-reactive with antifibronectin serum. Our results suggest that a restricted expression of the transformed phenotype and an increased adherence mediated by iododeoxyuridine correlates with an increased cathodic migration of fibronectin-like transformation-sensitive surface components in rat epithelioid and fibroblastic cells.

INTRODUCTION. Fibronectin is a polymorphic glycoprotein found in plasma, tissue and cultures of adherent vertebrate cells, which may mediate attachment of cells to a substratum. The known low level or absence of fibronectin from most but not all adherent transformed cells, may be responsible for the decreased anchorage or adherence of such cells (see 1-3, for Review).

Fibronectins produced by several transformed cells appear similar to normal fibronectin both in subunit composition (a dimer of 220,000 dalton subunits) and immunological characteristics (see 3, for Review). Since minor but significant differences may exist between "normal" and "transformed" fibronectins (3), we have now investigated this posibility in liver epithelioid cells known to possess a transformation-sensitive fibronectin (4) using two-dimensional mapping of iodinated cell surfaces which involves isoelectric focusing in the first dimension and sodium dodecyl sulphate-polyacrylamide gel electrophoresis in the second dimension (5).

0006-291X/81/060414-08$01.00/0

Since halogenated pyrimidines were earlier shown to change the tumorigenicity of cultured melanoma cells concurrent with a dramatic change in cell adhesiveness (6), and in view of the known involvement of fibronectin on cell adhesion (1-3), we now investigated whether growth of fibronectin-containing epithelioid cells in the presence of iododeoxyuridine affected their adhesiveness and isoelectric point of fibronectin. We now describe a novel effect of iododeoxyuridine in promoting the cathodic migration of fibronectin-like molecules in epithelioid cells concurrent with an effect on cell adherence, as well as a charge difference between "normal" and "transformed" fibronectin-like molecules.

## 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 (7,8). b) ts-24-NRK fibroblasts were derived by infection of normal rat kidney cells with the temperature-sensitive ts-24 clone of Rous sarcoma virus (kindly provided by Dr. John Wyke, ICRF, London). This cell line exhibited a flat morphology characteristic of well adhered normal fibroblasts when grown at 39° for 24 hours. When the same cells were grown below 36.5 °C for longer than 9 hours, the cells exhibit a typically transformed morphology consisting of weakly adherent cells, particularly after 12 hours at 33°.

Cultures were maintained in Roswell Park Memorial Institute Tissue Culture Medium 1640, supplemented with 10% fetal calf serum, unless otherwise indicated.

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 0.2% $KH_2PO_4$ (pH 7.2) with 5 mM glucose, using $1.8 \times 10^6$ cells per iodination for 10 min at 22°, as described elsewhere (4).

Immunoprecipitation with Anti-Fibronectin Serum and Analysis of Extracts. The antiserum used in the present study was prepared against purified fibronectin from normal baby hamster kidney cells and was kindly supplied by Dr. R.C. Hughes (National Institute for Medical Research, Mill Hill, London, England). The antigen was prepared by purification of the 230,000 dalton fibronectin-containing fraction with gelatin-containing Sepharose columns (9). The antiserum was found to give a single precipitation line against similarly prepared fibronectin from rat fibroblasts. Extracts from $1.8 \times 10^6$ iodinated 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 phenyl methyl sulfonyl fluoride, and 0.05 M Tris-HCl (pH 7.5) (4).

After removal of insoluble aggregates at 500 X g for 10 min, the supernatants (0.12 ml) were preabsorbed with 0.02 ml preimmune rabbit serum for 1 hour at 37° and further 16 hours at 4°. After cen-

trifugation at 12,500 X g for 20 min the supernatants were reacted with 0.02 ml of rabbit anti-fibronectin serum, for collection as described for the preimmune precipitates. Two dimensional assay of the iodinated extracts or immune precipitates was carried out in 8 M urea-1% Triton X-100 using pH 3.5-10 Ampholines in 5% polyacrylamide gels for the first dimension, followed by 7.5% Na DOD $SO_4$ polyacrylamide gel electrophoresis for the second dimension (5). In every case, simultaneous comparisons were carried out using 6.5 cm x 3 mm tubes for isoelectric focusing followed by parallel second dimensional electrophoresis in the same gel (10).

Iododeoxyuridine Effects and Cell Adherence Assays. Cultures were grown for 3 days in complete medium supplemented with 2.5 µg/ml iododeoxyuridine (IUDR) whenever indicated, and used for surface labelling (4) or adherence studies (11). The latter assays involved removal of traces of serum and use of serum-free medium for determination of the relative number of cells detached after a 10 min exposure of the cultures to 0.75 mM EDTA (11) or to 1 M urea (12) in phosphate-buffered saline (PBS) in both cases. After the 10 min incubation, duplicate plates were removed and the suspension containing detached cells collected for hemocytometer counting. The remaining cells were trypsinized for determination of the percentage of adherent cells (11).

## RESULTS

For the present study, we took advantage of the availability of the WIRL "normal" non-tumorigenic rat liver epithelioid line and its "transformed" tumorigenic derivative R72/3 cell line (7, 8) to extend reports from our laboratory on transformation-associated differences in surface macromolecules of epithelioid cell cultures (4). In agreement with our previous findings (4), "normal" rat epithelioid cells also exhibited most of their surface labelling in one major component which migrated in the 230K dalton fibronectin region at 3.7 cm from the cathode in addition to a trace of another more cathodic component of the same molecular weight approximately 2 cm from the cathode (Fig. 1A). In contrast, "transformed" epithelioid cultures showed significant labelling in the 60K, 140K and 230K regions, although the latter was always weaker than in the parent "normal" cells (4). An interesting feature of the labelling in the 230K region of "transformed" cells was the complete absence of the weakly labelled component with appeared about 2 cm from the cathode in the normal cells, as well as the expression of a weakly labelled component at a cathodic distance of 2.5 cm in addition to a more prominent spot about 3.45 cm from the cathode. Since halogenated pyrimidines are known to promote significant surface alterations and to increase cellular adhesion in mouse melanoma cells (6, 11), we thought it worthwhile to examine whether exposure to IUDR had any effect on fibronectin charge variants and cell adherence in fibronectin-positive WIRL epithelioid cells (4).

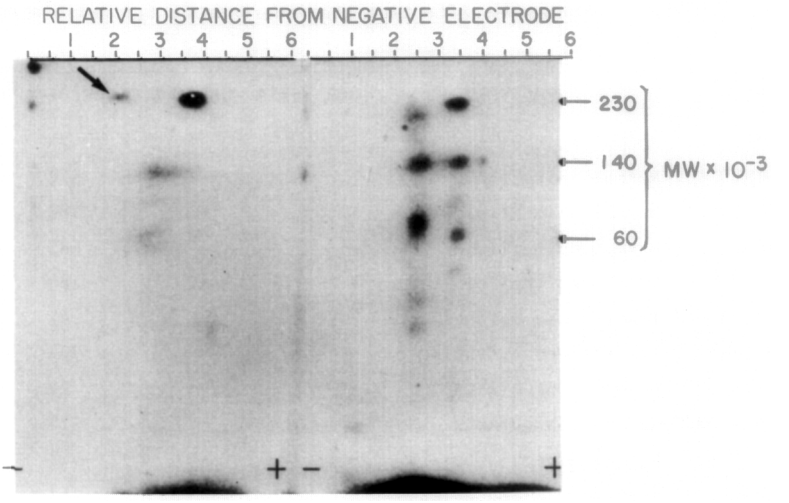


Figure 1. Two-dimensional Macromolecular Maps of Radioiodinated Normal and Transformed Rat Liver Epithelial Cells.
Cultures of WIRL normal and R72/3 transformed rat liver epithelial (4,6,7) cells were grown at 37° in RPMI 1640 medium supplemented with 10% fetal calf serum. After 24 hours, monolayers were washed and the medium was changed to a supplement of 10% fibronectin-deprived serum passed though gelatin-Sepharose (8) and cultures were grown for further 24 hours for iodination and two-dimensional analysis as described under Materials and Methods.
A. Pattern from control "normal" WIRL epithelial cells.
B. Pattern from control "transformed" R72 epithelial cells.

In agreement with reports on melanoma cells (6, 11), IUDR also led to a significant increase in WIRL cell adherence, as measured by cell detachment assays using 0.75 mM EDTA (11) or 1 M urea (12). The experiments in Table 1 showed that exposure to urea or EDTA led to much greater cell detachment in control than in IUDR-grown cells.

Since fibronectin is known to be a major cell surface protein involved in adhesion (see 1-3, for Review), we then investigated whether growth on IUDR also had an influence on surface-iodinatable components capable of immune precipitation with anti-fibronectin serum. For this purpose, surface-iodinated confluent cultures of WIRL cells were lysed, preabsorbed with preimmune serum and further reacted with antifibronectin serum for two-dimensional analysis of the immune precipitate (4). The results in Fig. 2 revealed in control cultures one major 220K component reactive with antifibronectin serum at about 3.5 cm from the cathode, similar to the major component iodinated in the subconfluent WIRL cultures shown in Fig. 1A. On the other hand, the corresponding immune precipitate obtained from iodinated extracts of WIRL cells

TABLE 1.- Conditions Affecting the Adherence of Epithelioid and Fibroblastic Cells

| CELLS | PHENOTYPE | IUDR | EDTA | UREA | % CELLS DETACHED |
|---|---|---|---|---|---|
| WIRL | Normal epithelioid | - | - | - | 2% |
| WIRL | Normal epithelioid | - | + | - | 53% |
| WIRL | Normal epithelioid | + | + | - | 18% |
| WIRL | Normal epithelioid | - | - | + | 68% |
| WIRL | Normal epithelioid | + | - | + | 18% |
| R72/3 | Transformed epithelioid | - | - | - | 4% |
| R72/3 | Transformed epithelioid | - | + | - | 73% |
| ts24 39° | Normal fibroblasts | - | - | - | 2% |
| ts24 39° | Normal fibroblasts | - | + | - | 52% |
| ts24 39° | Normal fibroblasts | - | - | + | 68% |
| ts24 33° | Transformed fibroblasts | - | - | - | 5% |
| ts24 33° | Transformed fibroblasts | - | + | - | 76% |
| ts24 33° | Transformed fibroblasts | - | - | + | 91% |

Epithelioid cell cultures were grown for 72 hours in medium plus 10% serum, containing 2.5 µg/ml IUDR whenever indicated. Fibroblasts were also grown in complete medium for 72 hours at either 39° (normal phenotype) or 33°(transformed phenotype) whenever indicated. Detachment was assayed in subconfluent cultures by removing the medium, washing monolayers with phosphate-buffered saline (PBS) and incubating the epithelioid cells with 0.75 mM EDTA in PBS (10) or to 1 M urea in PBS (11) for 10 min at 37°. Detachment assays with normal and transformed fibroblasts were carried out using 0.4 mM EDTA in PBS or 0.5 M urea in PBS. After the intervals indicated, duplicate plates were removed and the detached cells counted with a hemocytometer. The remaining adherent cells were similarly evaluated following a 10 min exposure to 100 µg/ml trypsin in PBS.

grown with IUDR, revealed a clearer expression of charge variants as well as a much more cathodic migration of the 220K fibronectin-like components which migrated at about 2 and 2.5 cm from the cathode (Fig. 2B).

Since the results presented above suggested that more adherent untransformed epithelioid cells showed a more cathodic 220K dalton fibronectin-like molecule, decreased in their transformed counterparts, we examined whether the same was true for phenotypically "normal" well adhered fibroblasts and phenotypically "transformed" less adherent fibroblasts (Table 1). The results in Fig. 3 showed that surface iodination of transformed fibroblasts led to some labelling in the 230K region as well as significant labelling in the 140K and 95K regions. Whereas the relative distance of the 230K fibronectin-like molecule of transformed fibroblasts was about 4 cm for the cathode, most of the

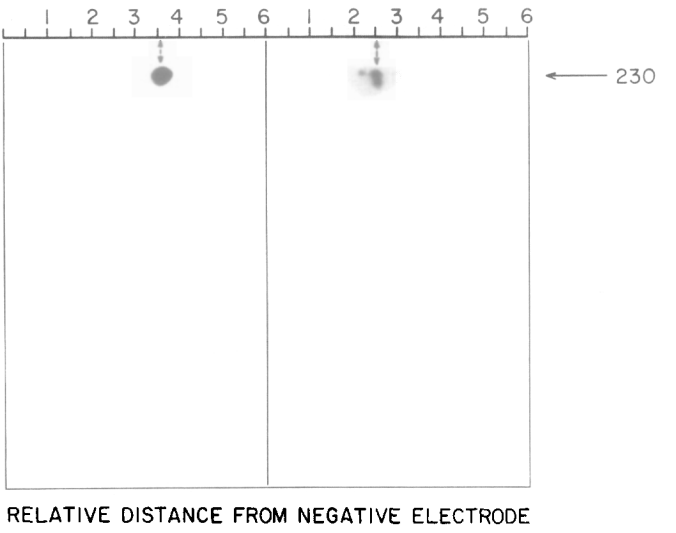


Figure 2. Influence of Iododeoxyuridine on the Anodic Migration of Epithelial Surface Fibronectins.
Replicate cultures of WIRL "normal" epithelial cells were grown for 36 hours at 37°, in medium with 10% complete fetal calf serum followed by further 36 hours in a medium supplemented with 10% fibronectin-deprived serum (8). Whenever indicated IUDR was added to a final concentration of 2.5 µg/ml throught the 72 hours incubation. Subsequently, subconfluent monolayers ($3.5 \times 10^6$ cells) were iodinated, lysed and immune precipitated with anti-fibronectin serum for two-dimensional analysis as indicated under Materials and Methods.
A. Pattern from, control cells.
B. Pattern from IUDR-treated cells.

other prominent surface proteins migrated closer to the cathode (3.2 cm). On the other hand, more adherent, phenotypically normal fibroblasts exhibited most of their surface labelling in a doublet in the 220K fibronectin region which migrated about 3.5 cm from the cathode, as well as trace labelling about 4 cm from the cathode.

## DISCUSSION

A comparison of surface-iodinated components of phenotypically normal and transformed fibroblasts by two-dimensinal mapping has now revealed that the expression of the transformed phenotype is associated with the well known decrease of a 220K major fibroblasts large external transformation-sensitive protein, also known as fibronectin (see 1-3). However, an additional difference between the phenotypically normal and transformed fibroblasts is the observation that the major 220K species of the normal fibroblasts is also more cathodic than the 220K species detectable in the less adherent transformed fibroblasts (Fig. 3, Table 1).

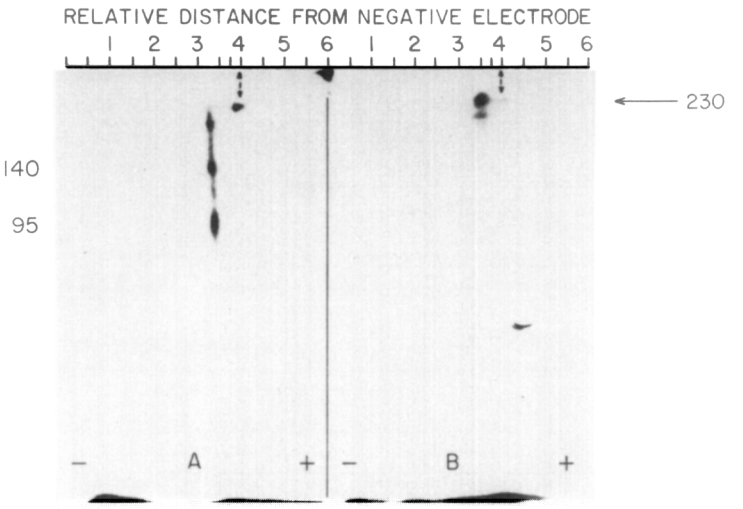


Figure 3. Two-dimensional Macromolecular Maps of Radioiodinated Normal and Transformed Fibroblasts.
Replicate cultures of normal rat kidney fibroblasts infected with the temperature-sensitive ts 24 clone of Rous sarcoma virus (kindly provided by Dr. John Wyke ICRF, London) were used for the two dimensional assays: subconfluent cultures of ($2.5 \times 10^6$) cells grown at 39° and 36° for 36 hours in medium with 10% fetal calf serum, were washed and grown for further 36 hours in medium supplemented with 10% fibronectin-deprived serum (8) for iodination and two-dimensional assays as described under Materials and Methods.
A. Pattern from transformed cells grown at 36°.
B. Pattern from phenotypically normal cells grown at 39°.

Our observations with fibroblasts indicating the presence of charge variants of fibronectin-like 220K transformation-sensitive surface components were also confirmed in liver epithelioid cells, and is compatible with previous data on plasma fibronectin heterogeneity (13). In this study, reaction of extracts from surface-labelled normal epithelioid cell with antifibronectin serum revealed the existence of 220K fibronectin-like charge variants, particularly in the case of cell cultures exposed to IUDR. A potentially more important observation than the influence of IUDR and *in vitro* transformation on the heterogeneity of the 220K dalton molecule is the apparent correlation that cultures that show more resistance to detachment by EDTA or urea also revealed more cathodic forms of surface-associated fibronectin-like molecules.

Since fibronectin is known to interact with collagen through hydrophobic bonds (14) and its interaction with cells may be ionic (15),

a "normal" cathodic fibronectin may play its role of mediating cell adhesion to a collagen substratum by partly neutralizing the cell surface anionic proteoglycans (16, 17).

## REFERENCES


1. Hynes, R.O. (1976) Biochim. Biophys. Acta 458, 73-107.
2. Yamada, K.M., and Olden, K. (1978) Nature 215, 179-184.
3. Vaheri, A., and Mosher, D.F. (1978) Biochim. Biophys. Acta 516, 1-25.
4. Rieber, M., and Rieber, M.S. (1980) Cancer Res. 40, 2562-2567.
5. Pena, S.D.J., Mills, G. and Hughes, R.C. (1979) Biochim. Biophys. Acta 550, 100-109.
6. Silagi, S., and Bruce, S.A. (1970) Proc. Natl. Acad. Sci. U.S.A. 66, 72-78.
7. Diamond, L., McFall, R., Tashiro, Y., and Sabatini, D. (1973) Cancer Res. 33, 2627-2636.
8. Steiner, A.F., Hentosh, P.M., Diamond, L., and Ting, R.C. (1977) Cancer Res. 37, 1864-1867.
9. Engvall, E., and Ruoslahti, E. (1977) Int. J. Cancer 20, 1-5.
10. Laemmli, U.K. (1970) Nature 227, 680-685.
11. Giotta, G.J., Brunson, K.W., and Lotan, R. (1980) Cell Biol. Int. Rep. 4, 105-116.
12. Vlodavsky, I., Johnson, L.K., and Gospodarowicz, D. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2306-2310.
13. Vuento, M., Wrann, M. & Ruoslahti, E. (1977) FEBS Lett. 82, 227-231.
14. Gold, L.I., and Pearlstein, E. (1980) E. Biochem. J. 186, 551-559.
15. Klebe, R.J. (1974) Nature 250, 248-251.
16. Kleinman, H.K., Mc Goodwin, E.B. and Martin, G.B. (1978) Ann. N.Y. Acad. Sci. 312, 436-438.
17. Linsenmeyer, T.F., Toole, B.P., and Gross, J. (1976). J. Cell Biol. 70, 59 (Abst).