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STUDIES ON THE IODINATED SURFACE MEMBRANE PROTEINS AND CONCANAVALIN A AGGLUTINATION OF TRANSFORMED SYRIAN HAMSTER CELLS

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Summary

Chemically transformed Syrian hamster cells exhibit marked agglutination in the presence of the plant lectin, concanavalin A. In this report, we describe conditions which can alter this concanavalin A agglutinability, and compare the surface proteins from transformed cells which express different degrees of agglutinability. Lactoperoxidase-catalyzed iodination of tertiary Syrian hamster cells reveals the major iodinatable protein to be approximately 220 000 daltons. The transformed Syrian hamster cells do not contain this protein in an iodinatable form. Analyses of the transformed cells grown under conditions which decrease the concanavalin A agglutinability do not demonstrate any iodination of the 220 000 mol. wt. protein. These results depict the effects of growth and dibutyryl cyclic AMP on the iodinatable cell surface proteins of transformed cells and indicate that the absence of the I-220 000 mol. wt. protein is probably not a major determinant of concanavalin A agglutination.

Introduction

Transformation of cells by oncogenic viruses and chemical carcinogens, as well as trypsinization or mitosis of normal cells, results in enhanced agglutination in the presence of certain plant lectins (for review, see ref. 1). It was subsequently observed that transformed cells frequently lack a large cell surface glycoprotein of approximately 200 000—250 000 mol. wt. [2—11]. This protein or group of proteins has been designated cell surface protein by Yamada and Weston [12], fibroblast surface antigen by Kuusela, Ruoslahti and Vaheri

Abbreviations: dibutyryl cyclic AMP, N^6O^2-dibutyryladenosine 3′:5′-monophosphate; EGTA, ethyleneglycol bis (β-aminoethylether)N,N'-tetraacetic acid.

[13]; large external transformation sensitive protein by Hynes [3] or Z by Blumberg and Robbins [4]. Since there is an increase in concanavalin A agglutinability of trypsinized cells and mitotic cells, and concomitantly a loss of the I-220 000 mol. wt. protein under these conditions [3,15—17], it has been suggested that this protein is affecting the agglutinability of cells. This protein or group of proteins might be responsible for constraints on the fluidity of membrane components which result in the relatively low agglutinability of untransformed cells. The loss of the I-220 000 mol. wt. protein would lead to increased agglutination [18—20].

To investigate the role of the large external transformation sensitive protein in agglutination of chemically transformed Syrian hamster cells, $A(T_1)Cl_3$, by concanavalin A, we have measured the agglutination after treatment with dibutyryl cyclic AMP and after replating the cells at varying cell densities. Concomitantly we have examined the sodium dodecyl sulfate polyacrylamide gel electrophoretic protein pattern and radioautographs of the cell surface proteins after iodination of the cells by the lactoperoxidase-catalyzed reaction.

We found that most of the iodinatable cell surface proteins do not change in the transformed cells during growth in the presence or absence of dibutyryl cyclic AMP. Also, these experiments suggest that even though the transformed cells can be modulated from a state of high agglutination by concanavalin A to levels comparable to untransformed fibroblasts by treatment with dibutyryl cyclic AMP [21] or by replating [22], they never express an iodinatable 220 000 mol. wt. protein on their cell surface. These results imply that the agglutination process is determined by factors other than this particular protein.

Materials and Methods

Primary cultures of Syrian hamster embryos were prepared and subcultured by twice splitting 1 : 5. These tertiary cultures were grown to confluencey in Eagle's minimal essential media (Grand Island Biological Company, Grand Island, New York) supplemented with amino acids, vitamins, and 5% fetal bovine serum. These cells were used as the control cells in the subsequent iodination and concanavalin A agglutination experiments. The transformed $A(T_1)Cl_3$ cells are a line of highly tumorigenic Syrian hamster cells which were cloned after exposure to 1-β-D-arabinofuranosylcytosine (a gift of Dr. W. Benedict, Los Angeles Children's Hospital, Los Angeles, Calif.). These cells produced fibrosarcomas when less than 1000 cells were injected subcutaneously into hamsters [23]. The $A(T_1)Cl_3$ cells have been subcultured many times in this laboratory and were grown in minimal essential medium containing 5% fetal bovine serum.

The tertiary Syrian hamster cells were grown to confluency, removed from the plates with 0.025 mM EGTA and tested for agglutination in the presence of 62.5 μg/ml concanavalin A (2 × crystallized, Miles Yeda, Kankakee, Ill.). The $A(T_1)Cl_3$ cells were plated at densities of $8 \cdot 10^5$ cells/60 mm plate in the presence or absence of 2 mM dibutyryl cyclic AMP. At the times indicated on Fig. 1, the cells remaining attached to the plates were iodinated or were removed from the plates, counted and tested for concanavalin A agglutinability. The concanavalin A agglutinability of $A(T_1)Cl_3$ cells was also measured at the same time intervals as the untransformed cells at initial cell densities given in Table I.

The method for testing concanavalin A agglutinability was essentially as described by Inbar and Sachs [24]. The cells were removed from the plate with 0.025 mM EGTA, washed and pelleted by centrifugation. A single cell suspension was made in phosphate buffered saline and 30 000 cells in 0.1 ml were mixed with 0.1 ml of 125 μg/ml of concanavalin A in plastic petri dishes for 10 min at 22° C. The agglutination was scored, using the grading described by Inbar and Sachs [24] by two observers.

Replicate 60 mm plates of the monolayer cells were iodinated by the lactoperoxidase-catalyzed method as described by Hynes [3]. The cells were washed with phosphate buffered saline 3 times prior to labeling. The labeling mixture contained 2 ml of phosphate buffered saline containing 5 mM glucose, 10 μl of 2 mM NaI in phosphate buffered saline, 10 μl of lactoperoxidase (5 mg/ml) obtained from Calbiochem, San Diego, Calif., 100 μl containing 1 mCi of $Na^{125}I$ (≈17 Ci/mg) purchased from New England Nuclear, Boston, Mass., and 10 μl of glucose oxidase (0.5 mg/ml) obtained from Calbiochem, San Diego, Calif.. After 10 minutes at 22° C the labeling solution was removed and the cells were washed in phosphate buffered saline with the NaCl replaced by NaI and containing 2 mM phenylmethylsulfonylfluoride as a protease inhibitor. The cells were removed from the plates and centrifuged at 1000 × *g* for 10 min. The pellets were lysed with 2% sodium dodecyl sulfate, 0.4 mM phenylmethylsulfonylfluoride in 0.1 M Tris · HCl (pH 7.4). The lysates were precipitated with two volumes of 100% cold acetone overnight at −20° C and then centrifuged at 10 000 × *g* for 30 min at 4° C. The precipitates were resuspended in sample buffer (0.14 M Tris · HCl (pH 6.8), 22.3% glycerol, 0.0011% bromphenol blue, 10% β-mercaptoethanol, 0.4 mM phenylmethylsulfonylfluoride 6% sodium dodecyl sulfate) and boiled at 95° C for 3 min. Equal amounts of trichloroacetic acid-precipitable ^{125}I were applied to either a 10 cm 5—15% polyacrylamide gradient slab gel containing 0.1% sodium dodecyl sulfate covered with a 4% stacking gel (Fig. 3) or a 10 cm 7.5% polyacrylamide gel covered with a 4.2% polyacrylamide gel stacking gel (Fig. 4) [25]. Gels were electrophoresed at 40 V for 22 h, stained with 0.1% Coomassie blue in 50% trichloracetic acid, dried into filter paper and autoradiographed on Kodak safety film.

Results

Effect of growth conditions on the agglutination of transformed cells by concanavalin A

In order to modulate the agglutinability of the $A(T_1)Cl_3$ cells, we replated them at varying densities after removal from tissue culture plates with 0.025 mM EGTA. At intervals after replating, the concanavalin A agglutination was monitored by the method and grading described by Inbar and Sachs [24]. When the $A(T_1)Cl_3$ cells were replated at an initial concentration of $8 \cdot 10^5$ cells/60 mm petri dish, the agglutinability was low (+) for up to three days. Fig. 1 demonstrates that the time required for these cells to regain maximal agglutinability (+++) was approximately seven days. By increasing the cell density at the time of replating, the lag phase for the re-expression of increased concanavalin A agglutinability was reduced (Table I). When $6 \cdot 10^6$ cells/60 mm petri dish were seeded, the lag phase for expression of increased concanavalin A

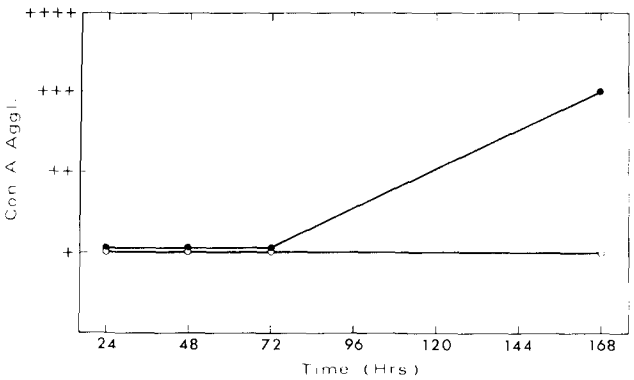

Fig. 1. Concanavalin A agglutination of $A(T_1)Cl_3$ cells at various times after replating of $8 \cdot 10^5$ cells per 60 mm plate; ●———●, $A(T_1)Cl_3$ cells; ○———○, $A(T_1)Cl_3$ cells grown in the presence of 2 mM dibutyryl cyclic AMP. The cells were removed from the plates with 0.025 mM EGTA, washed with phosphate buffered saline, resuspended at a concentration of 30 000 cells in 0.1 ml and incubated with 0.1 ml of concanavalin A at a final concentration of 62.5 μg/ml for 10 min at 22°C.

agglutination was reduced to between 48—72 h.

$A(T_1)Cl_3$ cells grown in the presence of 2 mM dibutyryl cyclic AMP were also examined for their agglutination by concanavalin A (Fig. 1). These studies revealed that growth in the presence of dibutyryl cyclic AMP reduced the agglutination to a 1+ level, which was comparable to untreated normal Syrian hamster fibroblasts. Other effects of the dibutyryl cyclic AMP were a decreased growth rate, a decreased final saturation density (Fig. 2), and a change in cell morphology from predominantly round cells to either elongated, spindle-shaped cells with long processes or polyhedral cells with a decreased nuclear to cytoplasmic ratio.

From these studies, it appeared that the agglutination of transformed cells could be modulated either by replating at selected densities or by growth in the presence of dibutyryl cyclic AMP.

Analysis of cell proteins and ^{125}I cell surface proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

In order to analyze the cell proteins and ^{125}I-surface proteins, we used the

TABLE I

EFFECT OF CELL DENSITY ON CONCANAVALIN A AGGLUTINATION

Cell no. ($\times 10^5$) [a]	Time (h) [b]		
	48	72	168
4	+	+	+++
8	+	+	+++
20	+	++	+++
40	+	++	+++
60	++	++	+++
80	++	++	+++

[a] Number of $A(T_1)Cl_3$ cells replated.

[b] Agglutination of $A(T_1)Cl_3$ cells by concanavalin A at various times after replating.

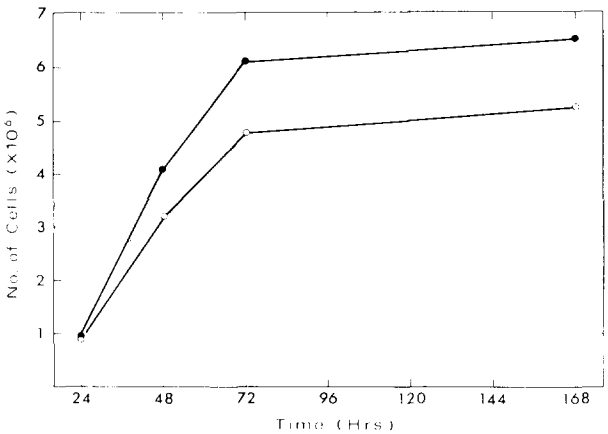

Fig. 2. Decrease in cell growth rate and saturation density by dibutyryl cyclic AMP; ●——●, $A(T_1)Cl_3$ cells; ○——○, $A(T_1)Cl_3$ cells grown in the presence of 2 mM dibutyryl cyclic AMP. The initial seeding density was $8 \cdot 10^5$ cells per 60 mm plate. Each point is the average number of cells counted from replicate plates.

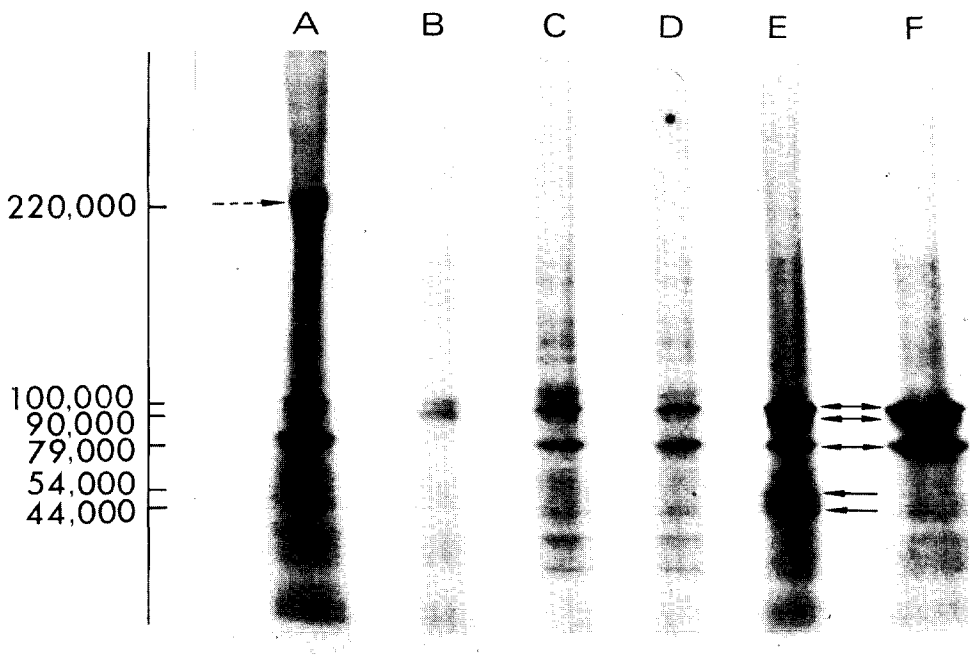

Fig. 3. Iodination of transformed and tertiary hamster cells. A. Tertiary Syrian hamster cells (arrow denotes 220 000 mol. wt. glycoprotein). B. Trypsinized tertiary Syrian hamster cells. C. Transformed Syrian hamster cells ($A(T_1)CL_3$) at 48 h after replating. D. Dibutyryl cyclic AMP-treated $A(T_1)Cl_3$ cells at 48 h after replating. E. Transformed Syrian hamster cells ($A(T_1)Cl_3$) at 168 h after replating (arrows denote the major alterations in labeling of the cell surface proteins). F. Dibutyryl cyclic AMP-treated $A(T_1)Cl_3$ cells at 168 h after replating. The labeling and electrophoresis of the cell proteins are described in Materials and Methods.

method described by Hynes [3]. Confluent cultures of tertiary Syrian hamster fibroblasts were labeled using the ^{125}I-lactoperoxidase-glucose oxidase catalyzed reaction and then lysed with sodium dodecyl sulfate. Equal amounts of trichloracetic acid-precipitable ^{125}I counts from each sample were loaded onto sodium dodecyl sulfate-polyacrylamide slab gels, electrophoresed and radioautographed (Fig. 3A). Treatment of the cells with 10 μg/ml trypsin for 10 min and subsequent iodination revealed that almost all the iodinatable proteins were removed (Fig. 3B); this implied that the conditions used for iodination were selectively labeling the cell surface proteins.

The A(T_1)Cl_3 cells (initially seeded at $8 \cdot 10^5$ cells/plate) grown in the absence or presence of dibutyryl cyclic AMP were iodinated and analyzed at 48 h and

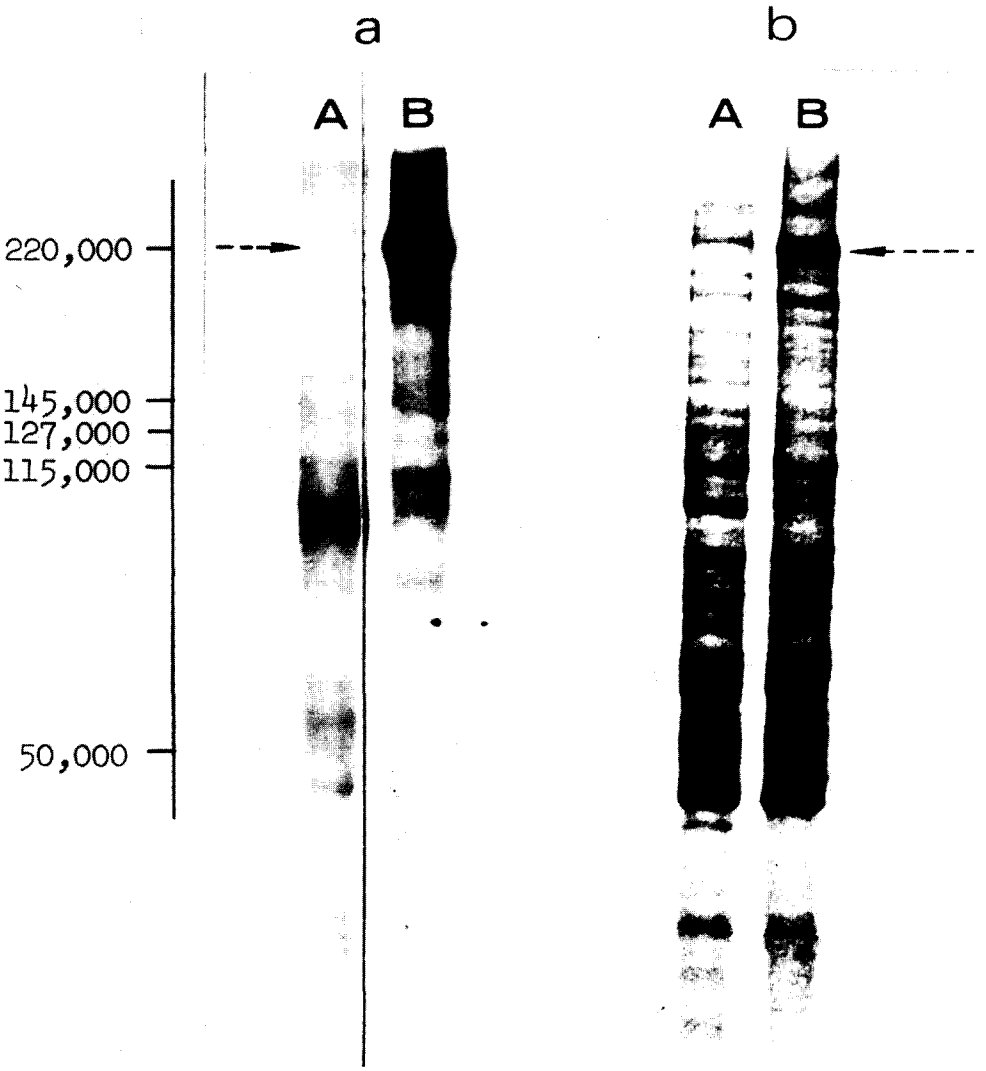

Fig. 4. Iodination (a) and Coomassie blue staining (b) of (A) A(T_1)Cl_3 cells and (B) tertiary hamster cells. The labeling and electrophoresis of the cell proteins is described in Materials and Methods.

168 h (Fig. 3C,D,E and F). Analyses of the radioautographs revealed that under none of these conditions is the 220 000 mol. wt. protein (which was present in the tertiary hamster fibroblasts) expressed in an iodinatable form on the $A(T_1)Cl_3$ cells. There were several differences in the profiles of the iodinated proteins occurring with time after replating or growth in dibutyryl cyclic AMP. With increased time in culture, the $A(T_1)Cl_3$ cells showed an increase in labeling proteins with approximate mol. wt. of 79 000, 90 000 and 100 000 (Fig. 3E and F). Without dibutyryl cyclic AMP in the medium, there were two additional iodinated proteins of approximately 44 000 and 54 000 mol. wt. which appeared on the $A(T_1)Cl_3$ cells (Fig. 3E).

Analyses of hamster fibroblast and $A(T_1)Cl_3$ cell lysates by Coomassie blue staining of the gel revealed that there was a protein co-migrating with the ^{125}I-labeled 220 000 mol. wt. protein that could be visualized in both the transformed and untransformed fibroblasts (Fig. 4b). This iodinatable 220 000 mol. wt. protein was not present in the autoradiograph of the transformed cells (Fig. 4a).

Discussion

The reason that transformed cells are more susceptible to agglutination by concanavalin A than normal cells has remained obscure despite numerous investigations. It was first suggested that tumor cells bind more concanavalin A than normal cells [26]. When it was shown that there was little difference in the amount of lectin bound by normal and transformed cells [27,28], it was proposed that this lectin induced the clustering of its binding sites into discrete patches. This clustering was thought to enhance the ability of these cells to adhere to each other [29]. However, it has been demonstrated that normal, non-agglutinating cells show clustering, and agglutinating malignant cells can show even dispersion of concanavalin A binding sites [30]. Willingham and Pastan [31] have proposed that agglutination is regulated through the modulation of cell surface microvilli by intracellular cyclic AMP levels.

The presence of a large molecular weight glycoprotein (250 000) has been detected on the surfaces of nonagglutinable cells. When these same cells were made agglutinable after trypsinization or after urea treatment, this protein was lost [17,32]. This protein was also absent or reduced in cells during mitosis [15,16] at which time they also increased in agglutinability [26]. Therefore, it has been proposed that this protein may play an important part in the agglutination phenomenon. This study was undertaken in an effort to correlate whether specific surface membrane protein alterations were correlated to agglutinability in a malignant cell system whose agglutination properties could be manipulated.

We have shown that these malignant cells, after replating, require several days of growth in order to regain full agglutinability. These results demonstrated a lag time for the restoration of the enhanced agglutination which has also been observed by Inbar et al. [22] to occur in other transformed Syrian hamster cells. Also, the treatment of the transformed cells with dibutyryl cyclic AMP resulted in a marked decrease in concanavalin A agglutination. Lactoperoxidase catalyzed ^{125}I labeling of the 220 000 mol. wt. glycoprotein was never detected

in the transformed cells; even when the agglutinability was markedly decreased. Therefore, it does not appear that the expression of this protein is directly controlling agglutination changes in this system. The observation that infection of certain mammalian cell lines with specific leukemia viruses can induce increased concanavalin A agglutinability with the loss of large external transformation sensitive protein [33] suggests that these surface membrane alterations are not necessarily interrelated. These experiments, as well as those reported in this paper, do not exclude the possibility that the loss of ^{125}I-220 000 mol. wt. protein is responsible for the increased agglutinability in some cells because it is possible that other factors such as growth of cells at low density or treatment with cyclic nucleotides may reduce the agglutinability by independent mechanisms.

The role in agglutination of some of the lower molecular weight surface proteins observed in highly agglutinable cells which are not present on cells treated with dibutyryl cyclic AMP remains to be assessed. In the culture allowed to grow to high densities (Fig. 3E) which demonstrates high agglutination with concanavalin A there are several changes in the proteins labeled. One of these proteins is of similar mol. wt. to actin and may represent some internal labeling in leaky cells or a difference in actin exposure to the external surface.

The presence of a 220 000 mol. wt. protein within or on the surface of transformed cells in a non-iodinatable form was suggested by analysis of the gel in Fig. 4. Studies using either iodination techniques or immunohistochemical labeling with an antibody against the 220 000 mol. wt. protein of 6 clones of chemically transformed lines of hamster cells have demonstrated either a marked decrease or absence of the ^{125}I-220 000 mol. wt, protein (Clarke, S. and Fink, L., unpublished). However, studies on chemically transformed mouse lines do not reveal any correlation between tumorigenicity and the absence of the 220 000 mol. wt. protein [34]. The reasons why many tumor cells are devoid of the iodinatable form of this protein are not understood. It was also thought that this protein could be involved in the regulation of cellular growth. However, studies by Teng and Cheng [35] and Zetter et al. [36] have shown that proteolytic removal of the 220 000 mol. wt. protein from the surface of resting cells does not correlate with proteolytic stimulation of DNA synthesis. Further studies on these ^{125}I-labeled proteins are necessary to determine their structural relationships to each other and to other membrane components, and to designate any physiological role for these proteins.

Studies by Yamada et al. [37] demonstrate that a cell surface protein released from chick embryo fibroblasts by treatment with 1 M urea agglutinates formalinized sheep erythrocytes in the presence of a divalent cation. They have suggested that the 220 000 mol. wt. glycoprotein under study may promote cell adhesiveness. Further studies are necessary to ascertain whether the loss of the ^{125}I-220 000 mol. wt. cell surface protein is related to the decreased adhesivity of tumor cells.

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