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ASYMMETRIC DISTRIBUTION OF SURFACE PROTEINS IN MONOLAYER CULTURE OF EMBRYONAL CARCINOMA F9 CELLS

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SUMMARY - Embryonal carcinoma F9 cells were labelled with [^{125}I]-lactoperoxidase either in monolayer culture or after their dissociation and also as dissociated multilayer aggregates. Two-dimensional gel electrophoresis analysis of iodinated proteins revealed two groups of surface proteins, characteristic of non-attached surface (group A) and of attached surface (group B). The content of group A proteins was 40.7 % in the case of monolayer culture and 10.2 % in the case of multilayer aggregates, as compared to the total value of their common surface proteins. With a direct method for detection of lectin-binding proteins it was shown that three major Concanavalin A-binding proteins belong to group A and one to group B. Two wheat germ agglutinin binding proteins were identified as surface proteins of group B.

INTRODUCTION - Recently, in our study of cell surface proteins in early embryogenesis of *Pleurodeles waltlii* (1) it was shown that difference exists between proteins exposed on the embryonic surface and those occuring in the region of cell-cell contacts. The protein pattern of the embryonic surface was not changed between the stage 8-32 cells and late gastrula, whereas the cell surface proteins from inside embryonic region were changed between early and late gastrula during the first morphogenetic movement. These results raised the question of whether the outside cells of embryo (which later become the endoderm) are entirely different from inside cells or the difference concerns only their non-attached surfaces, which are formed after detachement of the chorion. In other words, whether the attachement of cells from one side is followed by polarization of cell surface proteins.

We employed an embryonal carcinoma cell limited stem line F9 obtained from teratocarcinoma tumors of germ origin and cultured in monolayer. This enabled us to overcome the inaccessibility of the monolayer culture of embryonic

ABBREVIATIONS - EDTA, ethylenediaminetetraacetic acid ; 2D-electrophoresis, two-dimensional gel electrophoresis ; Con-A, concanavalin-A ; WGA, wheat germ agglutinin ; PBS, phosphate-buffered saline ; DFP, diisopropylfluorophosphat ; MW, molecular weight ; IP, isoelectric point.

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cells. The F9 stem cell line was first described by Bernstine *et al.* as nullipotent (2) while later Sherman and Miller have shown that F9 cells are able to form a small amount of endoderm (3). A differentiation of F9 cells is generated in the presence of retinoic acid (4). Previous studies have shown that the differentiation of embryonal carcinoma cells to their differentiated derivatives involves the important changes in cell surface proteins (5, 4).

Since cell attachment plays an important role in their differentiation, it was important to study its influence on the distribution of surface proteins. The aim of this work was to determine whether in monolayer culture cells exhibit differences between the proteins on the attached surface and the cell surface exposed to the medium.

MATERIALS AND METHODS

Cell cultures. Embryonal carcinoma F9 cells were plated in densities 10^6 per 10 cm diameter tissue culture dish coated with gelatin and cultured in Dulbecco's modified Eagle's medium supplemented with 15 % foetal calf serum in an atmosphere of 12 % CO_2 at 37°C for 48 hrs. In these conditions a confluent monolayer culture was obtained. The formation of round multilayer aggregates was achieved when the culture medium was changed twice every 48 hrs, otherwise F9 cells were incubated under the same conditions as described for monolayer culture.

Dissociation of cells. EDTA-mediated dissociation of cells was accomplished in PBS which was 1 mM in EDTA. The incubation was performed for 7 min at 22°C. Then PBS-EDTA was removed and the cells were detached with Hanks medium containing 5.5 mM glucose.

In the case of enzymic dissociation, the culture medium was replaced by 5 ml of Hanks medium containing 50 µg of pure Achromobacter collagenase of specific activity 1.8 µkat/mg (prepared in the laboratory). The attached cells were incubated 15 min at 37°C, then collagenase solution was withdrawn and the cells were detached with Hanks medium containing 5.5 mM glucose.

Iodination. The lactoperoxidase glucose/glucose oxidase procedure of iodination was used (6). All solutions used after cell dissociation were 1 mM in DFP.

Sample solubilisation. The samples were dissolved in 300 µl of lysis buffer containing 9.5 M urea 2 % (w/v) Nonidet P40, 2 % (v/v) ampholine and 5 % (v/v) 2-mercaptoethanol. Then 150 µl aliquots were applied to 2D-electrophoresis.

Two-dimensional polyacrylamide gel electrophoresis was done according to O'Farrell (7) using mixture of ampholines, pH range 3.5-10 and 4-6 (1 : 1). For pH measurement a control gel was cut into 0.5 cm pieces which were incubated with 1 ml of distilled water for 6 hrs. For the second dimension slab gels containing 10 % acrylamide and 0.17 % bis-acrylamide were used. The standard mixture applied in the second dimension contained β-galactosidase, phosphorylase, bovine serum albumin, aldolase (subunit) and chymotrypsinogen (MW 130 000, 96 000, 69 000, 40 000 and 25 000 respectively).

Staining, autoradiography. Gels were stained with Coomassie brilliant blue, dried and exposed with Kodak X-ray film.

Measurement of [^{125}I] incorporation. The developed X-ray films were superposed with corresponding Coomassie blue stained, dry gels. The position of iodinated spots on gels was marked. Then the identified spots were cutted out from dry gel and counted in a gamma counter.

Transfer of samples from 2D gels to nitrocellulose was done according to Bowen *et al.* (8). Unlabelled F9 cells were solubilized in lysis buffer and

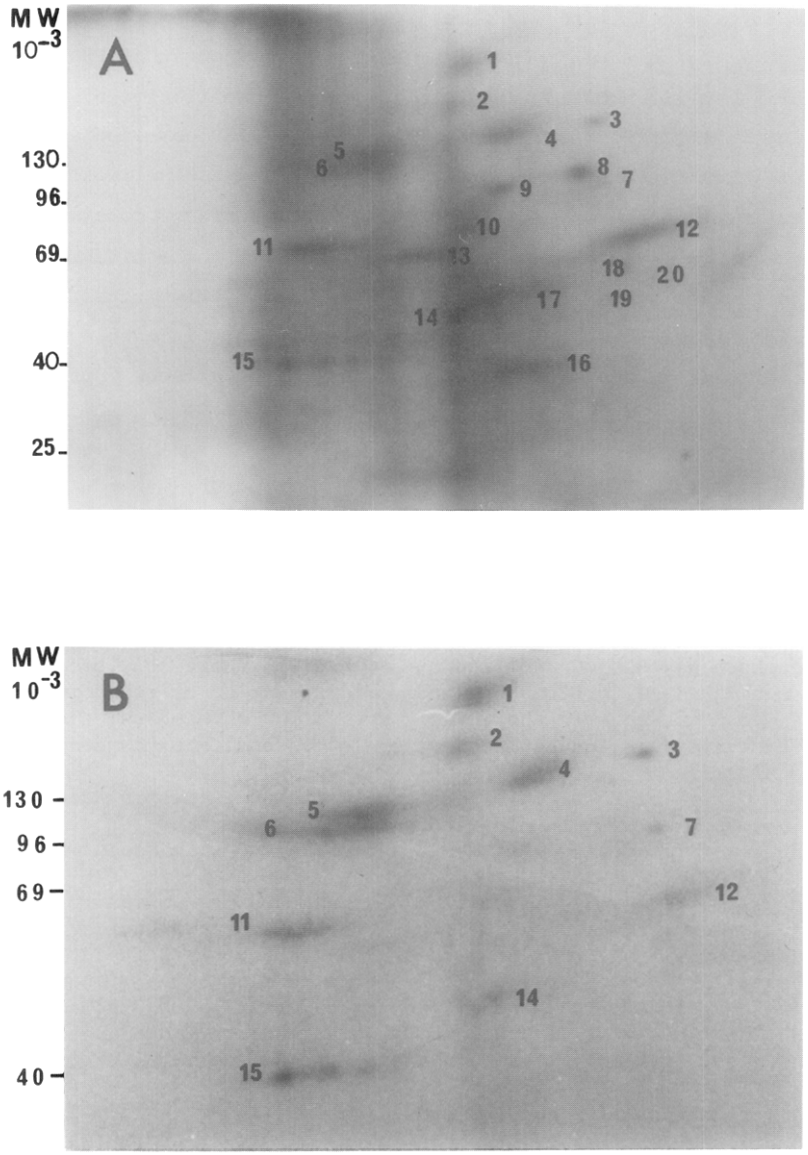

Figure 1. Two-dimensional gel electrophoresis of F9 cell surface proteins labelled with [^{125}I]-lactoperoxidase A) on EDTA-dissociated F9 cells, B) on monolayers of F9 cells attached to culture dishes, C) after dissociation of multilayer aggregates.

applied to 2D-electrophoresis. After staining, the samples were transferred to nitrocellulose.

Iodination of lectins. Concanavalin A and wheat germ agglutinin were obtained from Boehringer. The lectins (10 mg/ml in PBS) were iodinated with 2 mCi of [^{125}I] by lactoperoxidase method described above, in the presence of respective inhibitory monosaccharides (40 mg/ml).

Reaction with iodinated lectins was performed according to Burridge (9) using 4 % hemoglobin as carrier protein.

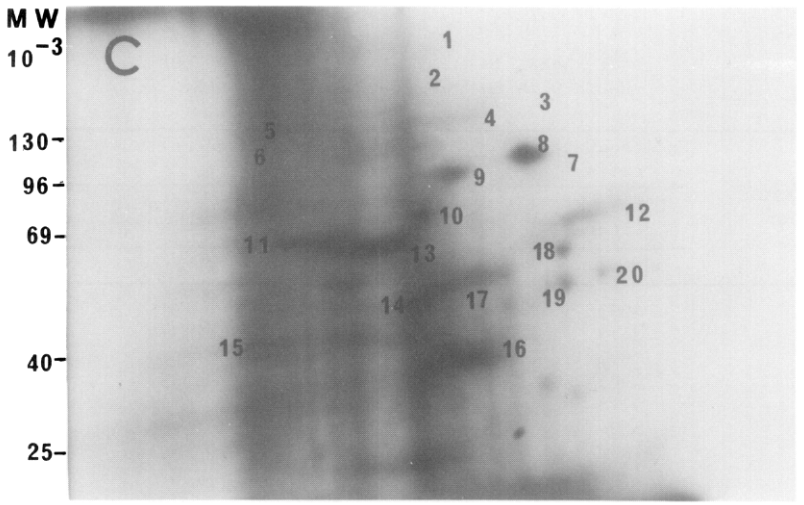

Fig. 1. (Continued)

RESULTS AND DISCUSSION - F9 cells were cultured for 48 hrs to obtain confluent monolayer, in two culture plates. In the first plate the cells were dissociated by EDTA, washed and labelled with [^{125}I]. The labelled cells were lysed and subjected to 2D-electrophoresis. Figure 1A shows the major [^{125}I]-labelled proteins which were iodinated on F9 cells dissociated by EDTA. In the second culture plate the cells were iodinated directly in the plate while they were attached to gelatin. The cells were then washed and detached. The following treatment was the same as described above. Figure 1B shows the pattern of proteins labelled on undissociated cells. As can be seen by comparing Fig. 1A and B, nine proteins (8, 9, 10, 13 and 16-20) that were labelled on EDTA dissociated cells were not labelled on undissociated cells.

To prove that these differences are not the artefacts of EDTA-treatment performed before iodination, the cells in monolayer culture were dissociated by pure Achromobacter collagenase and then treated and analysed under the same conditions as described previously. Collagenase from *Achromobacter iophagus* is a proteinase with high specificity for collagen or gelatin (the last one was used for coating of culture plates). Collagenase dissociation followed by iodination yields the same pattern of [^{125}I]-labelled proteins as in Fig. 1A. Therefore the proteins 8-10, 13, 16-20 occur on the attached surface and become available for lactoperoxidase iodination after dissociation of cells either by EDTA or collagenase.

As concerns the group of proteins 1-7, 11, 14, 15 labelled on undissociated cells (Fig. 1B), the extent of their labelling on dissociated and undissociated cells is rather similar (Table I). This indicates that in monolayer culture these proteins occur predominately on the non-attached surface. In

TABLE I

[^{125}I]-LABELLING OF SURFACE PROTEINS ON THE UNDISSOCIATED AND DISSOCIATED F9 CELLS

Protein number	Undissociated cells	Dissociated cells			
	Monolayer culture	Monolayer culture		Multilayer aggregates	
	cpm/spot	cpm/spot	% of total	cpm/spot	% of total
1	4073	3831	5.4	1915	1.0
2	2413	2535	3.6	1317	0.7
3	1897	2014	2.8	1007	0.5
4	4985	5272	7.4	3359	1.8
5	5118	5343	7.5	3774	2.0
6	5894	6115	8.6	3905	2.0
7	1050	882	1.2	521	0.3
8	-	3851	5.4	18824	10.0
9	-	3473	4.9	17752	9.4
10	-	2998	4.2	17489	9.3
11	3801	4765	6.7	18833	10.0
12	2105	5239	7.3	6554	3.5
13	-	5013	7.0	19994	10.6
14	3023	4215	5.9	18953	10.0
15	4148	2984	4.2	3513	1.9
16	-	4560	6.4	20315	10.7
17	-	5300	7.4	14841	7.8
18	-	1305	1.8	6924	3.7
19	-	1013	1.4	5690	3.0
20	-	592	0.8	3605	1.9
TOTAL	38507	71300	99.9	189090	100.0
TOTAL of non-attached surface proteins::			40.7		10.2

:: The values obtained for the proteins 11, 12 and 14 which are presumedly situated on both cell sides (see text) are not included.

the case of the protein 12 the value of [^{125}I] incorporation was 2.5 times higher after cell dissociation. This suggests that protein 12 is present on both sides of cell.

In the next experiment F9 cells were cultured until they formed confluent monolayer (48 hrs). Then the medium was changed twice, each 48 hrs. Under these conditions F9 cells form round multilayer aggregates. The results obtained with iodination of EDTA dissociated aggregates are shown in Figure 1C and Table I. It is evident that the amount of iodinated proteins characteristic for non-attached surface of F9 cells diminishes in the case of aggregates. On the contrary, a higher percentage of proteins 8, 9, 10, 13, 16-20, is available for [^{125}I]-labelling after cell dissociation.

Although the proteins 11 and 14 were labelled on non-attached surface, the level of their labelling increases in aggregates (Table I). Therefore,

like protein 12, we do not consider them as proteins characteristic of the surface exposed to the culture medium.

As can be seen in Table I the radioactivity incorporated into the proteins characteristic of the non-attached surface represents 40.7 % in the case of monolayer culture and only 10.2 % in the case of aggregates. This corresponds to the general decrease of ratio between non-attached and attached cell surfaces in multilayer aggregates.

All these results lead to the conclusion that one group of proteins (1-7, 13 and 15) occurs on the surface exposed to culture medium and another group (8, 9, 10, 16-20) on the attached surface of F9 cells.

For the further characterization of surface proteins belonging to each group, their affinity for different lectins was studied. For this purpose a method of direct detection on 2D-electrophoresis of lectin-binding proteins was developed. It consists of transfer of total cell proteins from 2D-gels to the nitrocellulose, followed by incubation with iodinated lectins. The advantage of this method is that cell lysis and 2D-electrophoretic analysis proceed under denaturating conditions where the post-transcriptional modifications like proteolysis or eventual neuraminidase action are essentially diminished. Moreover the samples for lectin treatment were prepared and analysed under the same conditions as used for the analysis of [^{125}I]-labelled surface proteins. This permitted the identification of Con-A or WGA-binding proteins in the total pool of [^{125}I]-labelled proteins, according to their position vis-a-vis other Coomassie blue stained proteins.

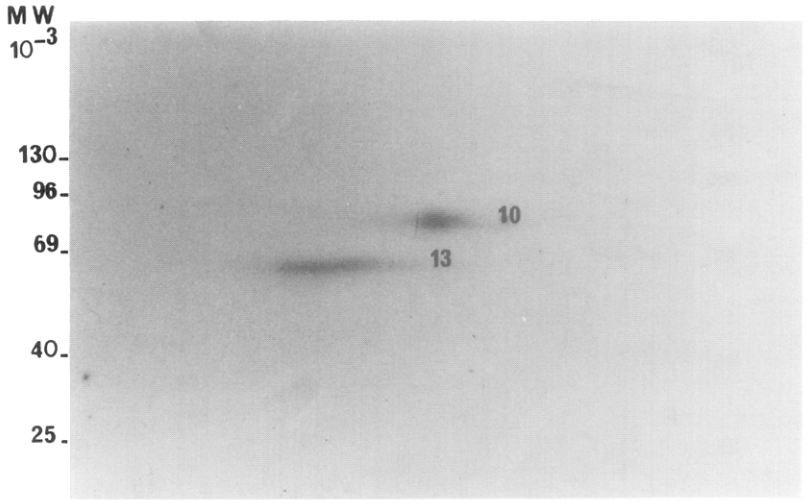

Figure 2. Identification on two-dimensional gel electrophoresis of WGA-binding proteins from F9 cells. The spots represent the radioactivity of bound [^{125}I]-WGA. The numbers indicate the position of corresponding surface proteins identified in Fig. 1A.

As it is shown in Figure 2 only two proteins of MW 80 000 and 68 000 bound [^{125}I]-labelled WGA. They have the same mobility as proteins 10 and 13 respectively (see Fig. 1). When the F9 proteins transferred to nitrocellulose were incubated with [^{125}I]-WGA in the presence of 2 % N-acetyl-D-glucosamine, then no iodinated spots were found. Therefore the reaction with [^{125}I]-WGA was specific.

As it was earlier reported, WGA has a high affinity for F9 cells and specifically agglutinates them (10). Our results show that WGA-binding proteins occur on the attached surface of F9 cells.

Con-A binding proteins were identified by the same procedure described for the WGA-binding proteins. The position of the major proteins binding [^{125}I]-Con-A is shown in Figure 3. Their migration in 2D-electrophoresis corresponds to that of proteins 1 and 3, 6 and 8 (Fig. 1A). The traces of labelled Con-A are also seen in the position of the protein 2 and below proteins 3 and 8. The traces of radioactivity below proteins 3 and 8 do not correspond to any of iodinated surface proteins. Therefore they can be considered either as artefacts or as glycoproteins which are not available for lactoperoxidase iodination. When the parallel sample was incubated with [^{125}I]-Con-A in the presence of 2 % α-methylmannoside, then no fixation of labelled lectin was detected.

The results obtained with Con-A have shown that among all surface proteins which have affinity for this lectin only protein 8 occurs on attached surface

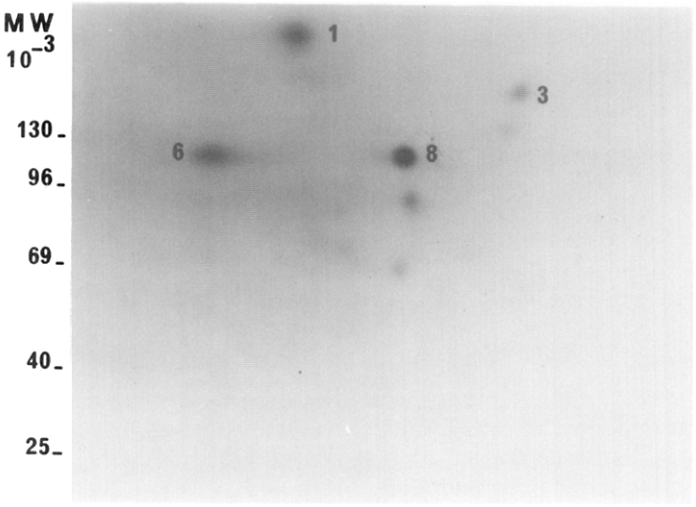

Figure 3. Identification on two-dimensional gel electrophoresis of Con-A-binding proteins from F9 cells.
The proteins were treated with [^{125}I]-labelled Con-A. The spots represent the radioactivity of bound [^{125}I]-Con-A. The numbers indicate the position of corresponding surface proteins identified in Fig. 1.

and the others (1,2,3 and 6) belong to the group identified on non-attached surface of F9 cells.

As it was previously reported, the Fab fragment of antibodies raised against F9 cells inhibited the cell-cell interactions in early mouse embryo (11). The main Fab target was found to be a Con-A-binding glycoprotein. Its tryptic fragment of MW 84 000 was isolated from embryonal carcinoma cells and from F9 tumors (12). A study of selective adhesion of embryonal carcinoma cells using Fab fragment from antibodies raised against F9 cells, has shown that [^{125}I]-labelled glycoprotein of MW ≈ 140 000 is responsible for cell-cell adhesion of teratocarcinoma cells (13). In the monolayer culture conditions we employed only one Con-A-binding protein of MW 120 000 (protein 8) was identified on the attached surface of F9 cells.

An importance of Con-A-binding proteins for the cell differentiation was emphasized by the fact that their profound redistribution on cell membrane takes place during myoblast differentiation (14). Moreover, recent studies of cell polarization in mouse embryo, using Con-A-binding assay, have shown that two distinct cell populations occupy different positions within the morula : the polar cells are peripheral and the apolar cells are central. Since peripheral and central cells give rise to trophectoderm and inner cell mass in the blastocyst it was suggested that polarization plays an important role in further embryonic differentiation (15, 16).

Our results summarized in Fig. 4 demonstrate that not only Con-A-binding proteins but also WGA-binding and other cell surface proteins are polarized

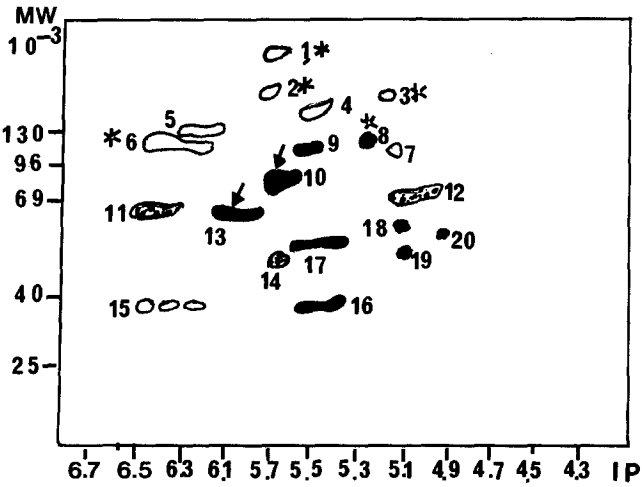

Figure 4. A schematic representation of F9 surface proteins characterized in this work.
○ - proteins identified on non-attached surface in monolayer culture ;
● - proteins originated from attached surface ; ⊙ - proteins which occur on attached and free surfaces ; ↙ - WGA-binding proteins ; * - Con-A binding-binding proteins.

on the membrane in monolayer culture of F9 cells. Furthermore the present work shows that cell-cell adhesion is not indispensible for this asymmetric distribution : monolayer of F9 cells attached to the gelatin-coated plates also exhibits polarization.

Therefore the presence of attached and non-attached surfaces in cell lead to the profound asymmetric distribution of membrane proteins and probably of other membrane components.

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