Biochimica et Biophysica Acta, 734 (1983) 249–256
Elsevier

BBA 71842

CHANGES IN PATTERN AND ACCESSIBILITY FOR ^{125}I-LABELLING OF CELL-SURFACE PROTEINS AFTER MESENCHYMAL DIFFERENTIATION OF EMBRYONAL CARCINOMA CELLS

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(Received May 16th, 1983)

Key words: Cell surface; Membrane protein; Protein iodination; Concanavalin A-binding protein; Differentiation; (Mouse embryonal carcinoma)

Cell-surface proteins of the embryonal carcinoma line C17-S1 1003 (*1003*) and of some of its mesenchymal derivatives were studied. The surface proteins were labelled with ^{125}I using the lactoperoxidase-glucose-glucose oxidase system either on the cells attached to the culture dishes or after their dissociation. Iodinated proteins were analyzed by two-dimensional gel electrophoresis. The patterns obtained with embryonal carcinoma cells *1003* and with two mesenchymal cell types derived from them, namely embryonic mesenchymal cells (line *10035*) and fibroblastic cells (line *10031*), were different one from the other, especially when considering the group of proteins labelled on the attached cells. The pattern of cell-surface proteins of the myoblastic line *1168*, also derived from C17-S1, was found to be similar to that of *10031* fibroblastic cells. This result is discussed in the light of the phenotypic transition toward myogenesis, which can be obtained with *10031* fibroblastic cells but not with *10035* embryonic mesenchymal cells. A direct method of detection of lectin-binding proteins permitted us to identify the major concanavalin A-binding proteins. Two of them are common to all cell lines studied. They were labeled with ^{125}I on the attached undifferentiated *1003* cells, while in all differentiated derivatives they became available for labelling after the cell detachment only.

Introduction

Cell-to-cell communication occurs either via diffusible factors or by contact with neighbouring cells and extracellular matrices. In all cases, it involves the surface of the cells. For this reason the plasma membrane is thought to play a major role in the co-ordinated events of differentiation occurring during embryonic development. Because of technical problems, it is difficult to undertake a functional study of the plasma membrane in the embryo. The teratocarcinoma of the mouse has proven to be a convenient system for the study of the early steps of development in vitro [1–5].

Changes in cell-surface proteins occur during early embryogenesis [6] as well as during differentiation of embryonal carcinoma cells [7,8]. Furthermore, a quantitative comparison of the proteins identified either on the attached or dissociated cells in subconfluent cultures of the embryonal carcinoma line *F9* revealed their irregular distribution [9]. Such irregularity might be involved in the control of differentiation exerted by attachment in this cell line [10].

It was previously shown that pathways of differentiation of the embryonal carcinoma cell line C17-S1 1003 (*1003*) are dependent on both soluble factors and attachment factors [11,12].

The aim of the present work was to investigate the changes of cell-surface proteins available for ^{125}I-labelling on attached and dissociated cells after several pathways of mesenchymal differentiation

had been completed by embryonal carcinoma cells. Embryonic mesenchymal cells, fibroblasts and myoblasts were compared to embryonal carcinoma cells.

Materials and Methods

Obtention of cell lines and tissue culture methods have been described previously [11,13].

Morphology, developmental potential and tumorigenicity of the cells lines. The following cell lines were used in this study: *C17-S1 1003 (1003)*, an embryonal carcinoma cell line, derived from C17-S1 [11]; it has a typical embryonal carcinoma morphology (Fig. 1A) and produces teratocarcinomas when injected into syngeneic animals (Fig. 2A). It is able to differentiate in vitro into nerve cells, embryonic mesenchyme, fibroblasts and muscle [11,12,14,15,20]. *10035 Clone C1 (10035)*, an embryonic mesenchymal cell line derived from *1003* [14]; *10031 Clone D4 (10031)*, a fibroblastic cell line derived from *1003* [15]; *1168*, a myogenic cell line derived from the parental C17-S1 line [13].

10035, 10031 and *1168* have all a fibroblastic morphology in exponential cultures (Fig. 1B, C, D). *10035* does not further differentiate in vitro. *10031* does not differentiate in vitro spontaneously, but may be induced to form muscle tissue by treatment with 5-azacytidine or adipose tissue after treatment with dexamethasone [15]. *1168* forms myotubes in vitro [13]. *10035* forms embryonic mesenchymal tumours (Fig. 2B) and *10031* forms fibrosarcomas after injection into mice (Fig. 2C), whereas *1168* is not tumorigenic.

Cell dissociation. In order to dissociate cells with EDTA, the culture medium was removed and

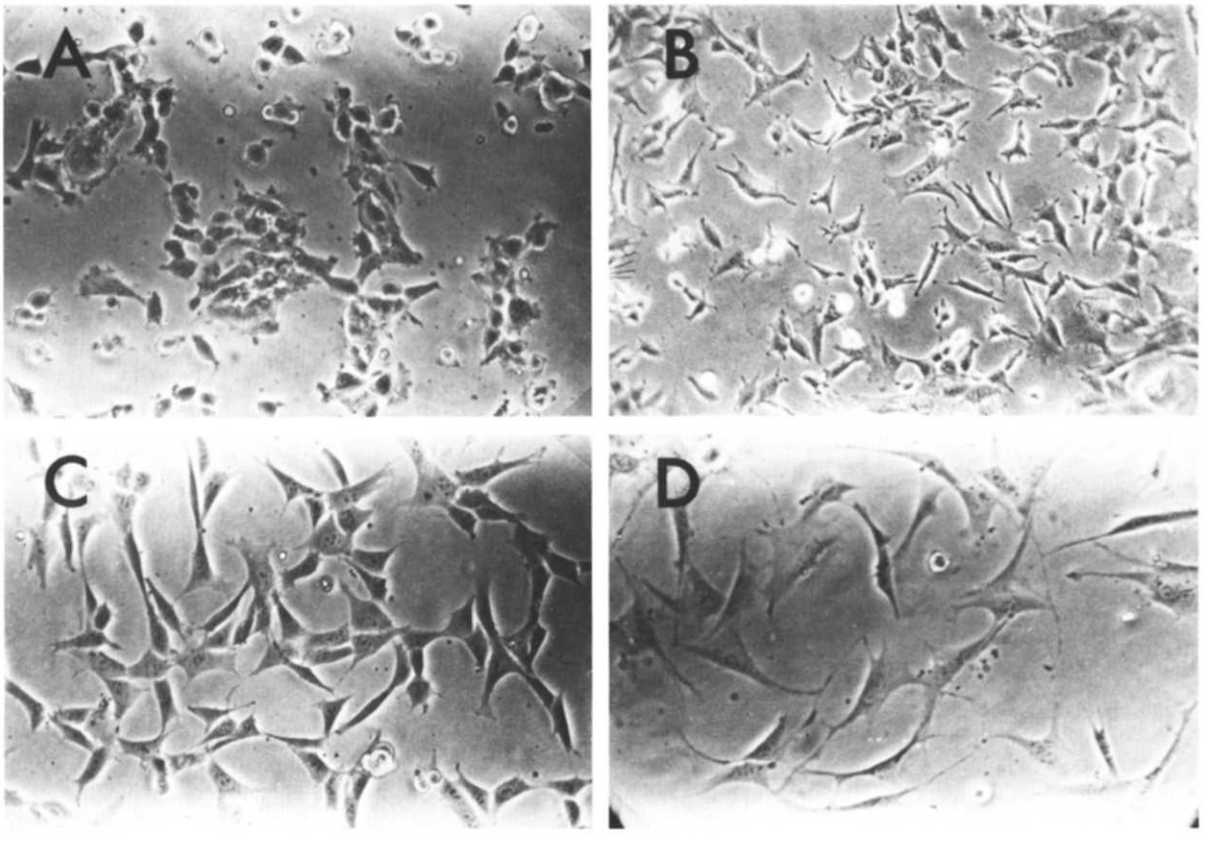

Fig. 1. Phase-contrast photomicrographs of embryonal carcinoma *1003* cells and their mesenchymal derivatives. (A) *1003*; (B) *10035*; (C) *10031*; (D) *1168* cells.

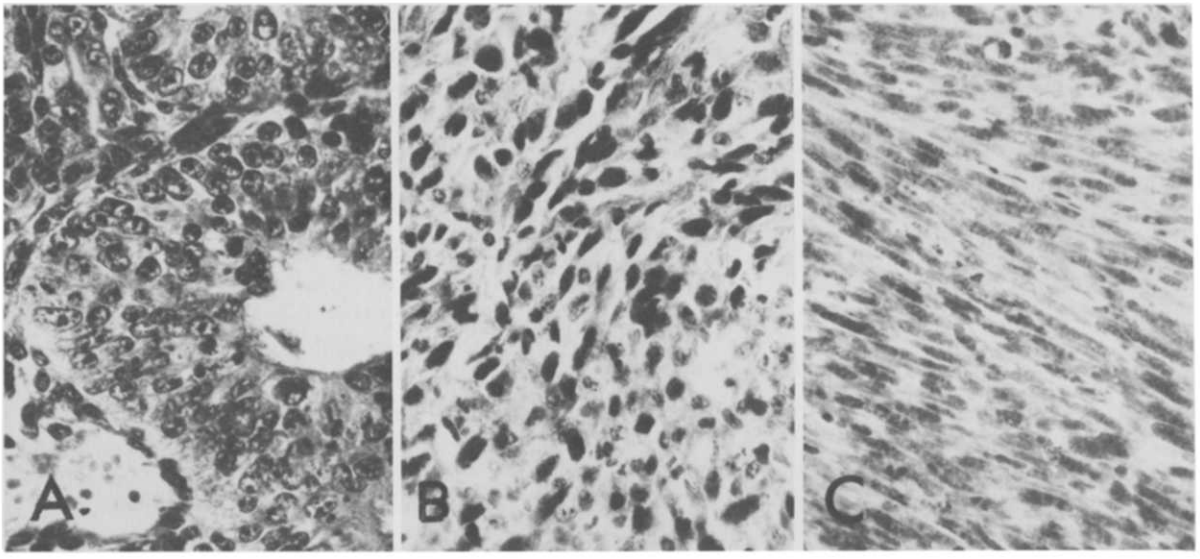

Fig. 2. Sections of tumors formed by *1003* cells and by their mesenchymal derivatives. (A) *1003*, teratocarcinoma; (B) *10035*, embryonic mesenchymal tumor; (C) *10031*, fibrosarcoma.

10 ml of buffer A (0.2 M NaCl/3.2 mM Na_2HPO_4/3.4 mM KH_2PO_4/2.6 mM KCl (pH 7.4)) which was 1 mM in EDTA (buffer B) were added. After incubation for 7 min at 22°C, buffer B was removed and the cells were detached with Hanks' medium containing 5.5 mM glucose.

Iodination. The lactoperoxidase-glucose-glucose oxidase procedure of iodination was used [16]. Cells attached to the culture plate were covered with 5 ml of Hanks' medium which was 5.5 mM in glucose. The cells dissociated by EDTA were suspended in 5 ml of the same medium. Then 50 μg of lactoperoxidase (67 U/mg, Sigma), 1 mCi of carrier-free ^{125}I, sodium salt (100 Ci/l, Amersham) and 10 μg of glucose oxidase (277 U/mg, Sigma) were added. The iodination was performed at room temperature for 20 min. Attached cells were washed three times with Hanks' medium after iodination, then detached in buffer B as described above. All samples were washed with 15 ml of Hanks' medium supplemented with 150 mM unlabelled KI. All solutions used after cell dissociation were 1 mM in diisopropylfluorophosphate (DFP).

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

Two-dimensional polyacrylamide gel electrophoresis. This technique was applied according to O'Farrell [17] using a mixture of ampholines of pH range 3.5–10 and 4–6 (1 : 1). A control gel was cut into 0.5 cm slices which were incubated with 1 ml of distilled water for 6 h, and the pH was measured with a Metrohm Herisau pH meter. For the second-dimension slab gels containing 10% acrylamide and 0.17% bisacrylamide were used. The standard mixture applied in the second dimension contained β-galactosidase, phosphorylase, bovine serum albumin, aldolase (subunit) and chymotrypsinogen (M_r 130000, 96000, 69000, 40000 and 25000, respectively). Gels were stained with Coomassie brilliant blue, dried and exposed with Kodak X-ray film (Kodirex) for 4–6 days.

Transfer of samples from two-dimensional gels to nitrocellulose. Unlabelled cells were solubilized in lysis buffer and applied to two-dimensional electrophoresis. After staining, the samples were transferred to nitrocellulose according to Bowen et al. [18].

Iodination of Concanavalin A and its reaction with transferred proteins. 10 mg of concanavalin A (Boehringer) in 1 ml of buffer A were iodinated with 2 mCi of $Na^{125}I$ by the lactoperoxidase

method described above in the presence of methyl α-mannoside (40 mg/ml). The reaction mixture was diluted up to 5 ml with buffer A which was 150 mM in unlabelled KI and dialyzed twice against 500 ml of buffer A at 4°C during 24 h. Reaction of proteins transferred on nitrocellulose sheets with the iodinated lectin was performed according to Burridge [19] using 4% hemoglobin as saturation protein.

Results

Comparison of surface protein patterns of 1003, 10035, 10031 and 1168 cell lines

Each cell line was grown in two culture plates to obtain a subconfluent monolayer (Fig. 1). Then lactoperoxidase-catalyzed ^{125}I-labelling of surface proteins was performed either on the cells still attached to the culture plate or after their dissociation. The cells were lysed and their proteins were separated by two-dimensional electrophoresis. A comparison of ^{125}I-labelled surface proteins of *1003* and *10035* cells is shown in Fig. 3.

It can be seen that the patterns of major proteins labelled on the attached cells of both cell lines are different (Fig. 3A and C). The differences consist in the availability of the proteins *4, 9, 14* and minor proteins *2, 4–6, 23–26* for labelling on the attached mesenchymal cells *10035*, and the absence of labelling of the proteins *12, 17* and *18–20*.

Another group of proteins was found to be

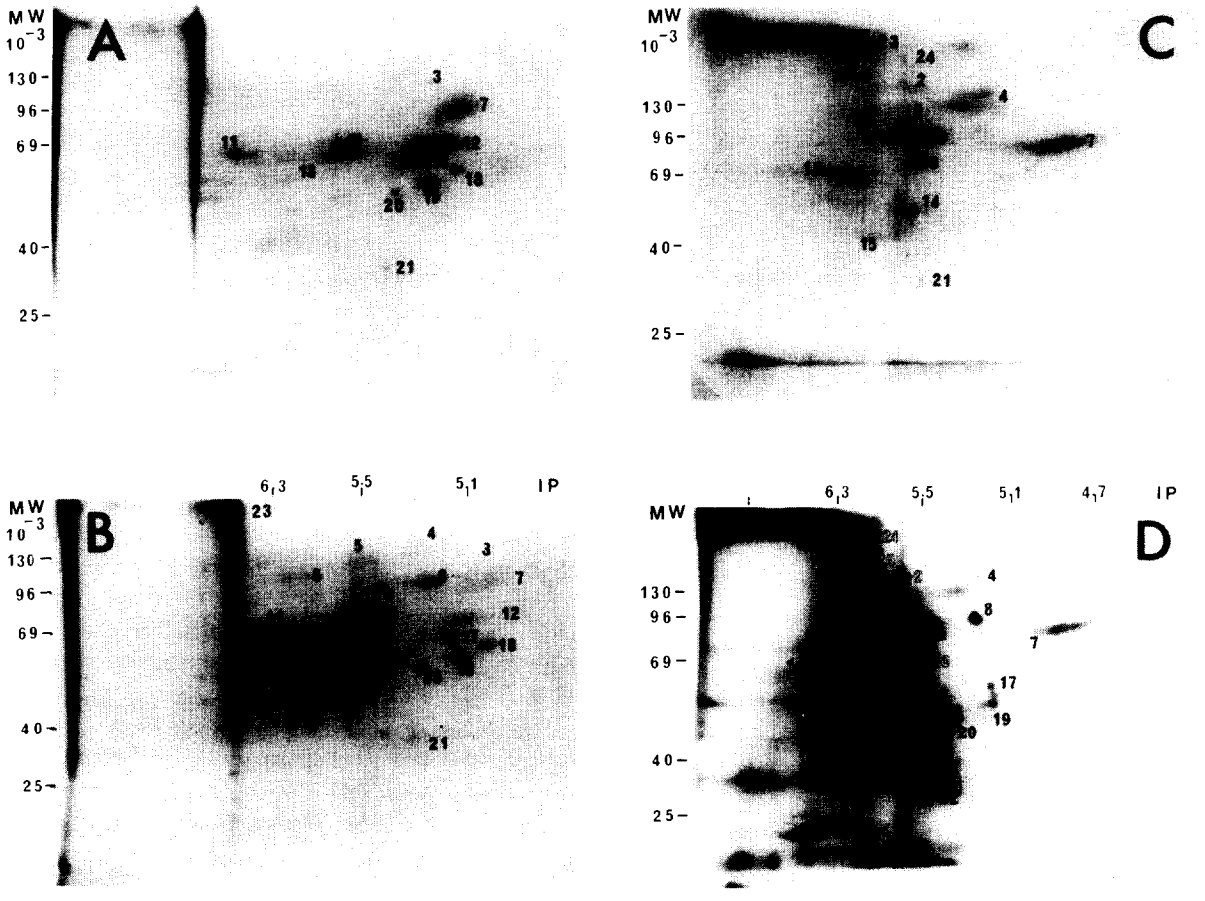

Fig. 3. Two-dimensional gel electrophoresis of cell-surface proteins labelled with ^{125}I: (A) on monolayer of embryonal carcinoma *1003* cells attached to culture dish; (B) on dissociated *1003* cells; (C) on monolayer of mesenchymal *10035* cells; (D) on dissociated *10035* cells.

labelled after the cell detachment only (Fig. 3B and D). In the case of *1003* cells, the proteins *8*, *9* and *13–15* as well as the traces of proteins *4–6* and *23, 28* were labelled under these conditions.

As shown in Fig. 3D, the pattern found after detachment of mesenchymal *10035* cells is different from that obtained with *1003* cells. Apart from quantitative differences especially in proteins *9, 11, 14, 15* and *21*, the main qualitative difference consists in the appearance of protein *22*.

Two-dimensional electrophoretic analysis of iodinated proteins of *10031* and *1168* cells are compared in Fig. 4. It can be noted, that the patterns of proteins labelled on the attached cells as well as those revealed only after cell dissociation show a high degree of similarity. The only difference is that proteins *12, 21* and *28* are labelled on attached fibroblasts *10031* (Fig. 4A) but not on attached myoblasts *1168* (Fig. 4C). And on the other hand, a high level of protein *11* is labelled on attached myoblasts.

The results in Fig. 4 show a high content of proteins in the pH range 5.6–6.5 and molecular weights 120 000–200 000 labelled on the attached fibroblasts *10031* and myoblasts *1168*. These proteins are found only as traces on the attached *10035* mesenchymal cells (Fig. 3C), and they are absent on attached embryonal carcinoma *1003* cells (Fig. 3A). In this respect, *10035* mesenchymal cells are intermediate between *1003* on one hand, and *10031* fibroblasts and *1168* myoblasts on the other hand.

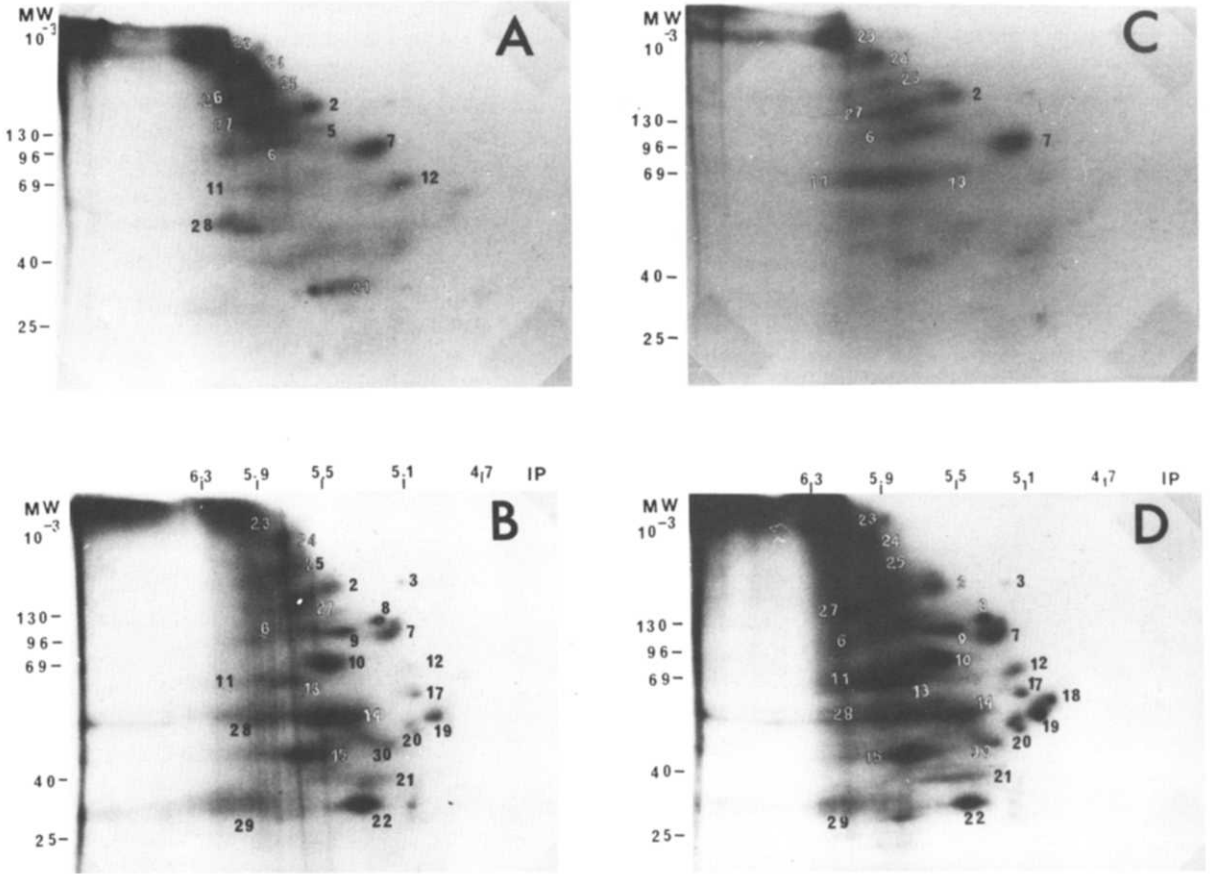

Fig. 4. Two-dimensional gel electrophoresis of cell-surface proteins labelled with ^{125}I: (A) on monolayer of fibroblastic *10031* cells (type 2); (B) on dissociated *10031* cells; (C) on monolayer of myoblastic *1168* cells; (D) on dissociated *1168* cells.

In contrast, after detachment of all differentiated cell lines the patterns of labelled proteins (except protein *4*) show a high degree of similarity (Fig. 3D and 4B, D). As concerns undifferentiated *1003* cells, their pattern (Fig. 3B) shows quantitative and qualitative differences in comparison with that of differentiated derivatives. The main difference concerns the high-molecular-weight proteins in the pH range of 5.6–6.5: they are only in trace amounts on undifferentiated *1003* cells. Also, protein *22* is labelled only on the differentiated cell derivatives.

In spite of above-mentioned differences in the patterns of detached cells we found generally that the most important changes after differentiation of embryonal carcinoma *1003* cells occurred among proteins which are available for ^{125}I-labelling on the cells attached in monolayer culture. This concerns in particular proteins *18–20*: they are labelled on the attached *1003* cells (Fig. 3A), whereas in all differentiated derivatives they are labelled after the cell detachment only.

Identification of major concanavalin A-binding proteins

The proteins of each cell type were transferred

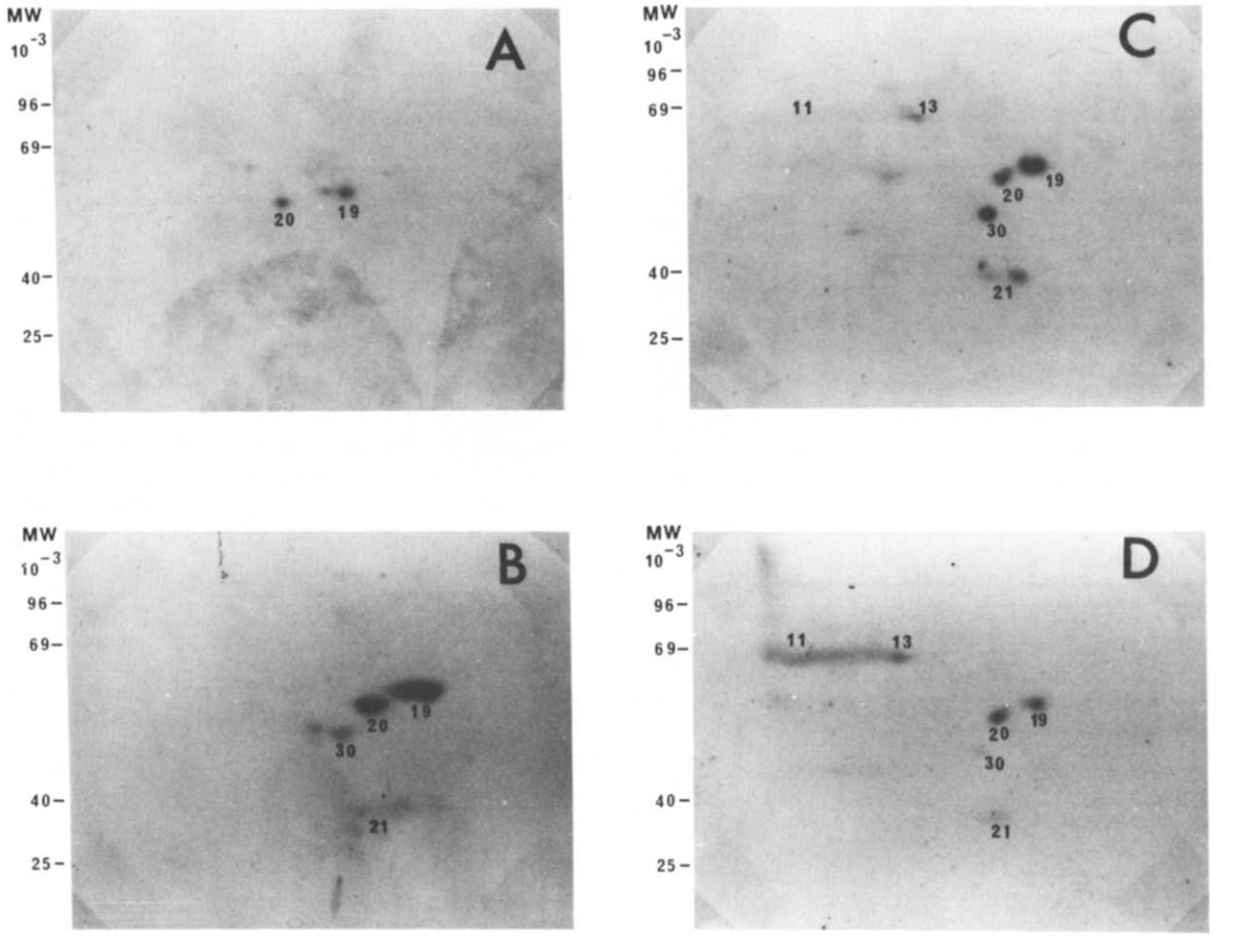

Fig. 5. Identification of concanavalin A-binding proteins on two-dimensional gel electrophoresis. The proteins were treated with ^{125}I-labelled concanavalin A. The spots represent the radioactivity of bound ^{125}I-labelled concanavalin A on the proteins from: (A) *1003*; (B) *10035*; (C) *10031*; (D) myoblastic *1168* cells. The numbers indicate the position of corresponding surface proteins identified in Fig. 3 and 4.

after their separation by two-dimensional electrophoresis to nitrocellulose sheets which were subsequently incubated in the presence of ^{125}I-labelled concanavalin A. The position of lectin-binding proteins was determined according to the radioactive spots of retained labelled concanavalin A. A comparison of the relative positions of concanavalin A binding proteins on one hand and of proteins stained with Coomassie blue (not shown) and lactoperoxidase-labelled surface proteins on the other hand permitted us to identify which of all surface proteins have affinity for concanavalin A.

The results of Fig. 5 show that two major concanavalin A-binding proteins, *19* and *20* were identified in the four studied cell lines. In addition, ^{125}I-labelled concanavalin A was bound by proteins *30* and *21* in all differentiated derivatives.

A comparison of Fig. 3, 4 and 5 shows that concanavalin A-binding proteins of *1003* embryonal carcinoma cells belong to the group of proteins available for ^{125}I-labelling on the attached cells; in contrast, in all differentiated cell lines (*10035, 10031* and *1158*) these proteins belong to the group of surface proteins which are labelled after cell detachment only. Therefore, the changes in accessibility of these concanavalin A-binding proteins for lactoperoxidase-catalyzed iodination take place after all studied pathways of mesenchymal differentiation.

Other concanavalin A-binding proteins, proteins *11* and *13*, were found in cell lines *1168* and *10031* only. In the latter, protein *11* was present as traces. It must be noticed that although proteins with the electrophoretic mobilities of proteins *11* and *13* are present on the medium-exposed surface of *1003* and mesenchymal *10035* cells (Fig. 3A, C), they do not show affinity for concanavalin A. It is difficult to decide whether these proteins are different or whether the difference concerns their glycosylated moiety only.

Discussion

Results presented here show that changes in the pattern and the accessibility for ^{125}I-labelling of cell-surface proteins occur after differentiation of embryonal carcinoma cells into various mesenchymal derivatives (embryonic mesenchyme, fibroblasts, myoblasts). After cell detachment, the appearance of a new group of proteins available for iodination cannot be considered as an artefact introduced by EDTA treatment: after labelling, the attached cells were also treated with EDTA under the same conditions. Therefore, these proteins are not available for the lactoperoxidase-catalyzed iodination on the attached cells either because of steric hindrance or because of their direct involvement in the mechanism of attachment. On the contrary, the proteins labelled on the attached cells are in contact with culture medium. A comparison of Fig. 3 and 4 shows that the most significant changes after different pathways of differentiation of EC *1003* cells occur particularly in this group of proteins. In this respect, mesenchymal *10035* cells display an intermediate pattern between *1003* embryonal carcinoma cells and *10031* fibroblasts or *1168* myoblasts.

As was previously shown, the myogenic clonal cell lines exhibit phenotypic instability [13], in other words, the ability of the cell to change (generally revesibly) its phenotype. Such transitions were shown to be dependent on the environmental conditions and are often observed between fibroblasts and myoblasts.

In the present study, a high degree of similarity between cell-surface proteins of fibroblastic and myoblastic cells was demonstrated (Fig. 4B, D). Nevertheless, some differences in their medium-exposed proteins must be noticed (Fig. 4A, C). This concerns mainly the proteins *12, 21* and *28*. They are exposed to medium on fibroblastic cells *10031* only (Fig. 4A, C). Also, the amount of the medium-exposed concanavalin A-binding protein *11* on these two cell lines is different. Therefore, the main difference between myoblastic and fibroblastic cells concerns the proteins exposed to the culture medium. This fact could be important for a future phenotypic determination of cells.

A feature common to all differentiation pathways studied consists on one hand in the appearance of high-molecular-weight proteins *4–6* and *23–27* on the surface exposed to the culture medium and on the other hand in the disappearance from it of protein *17* and also of concanavalin A-binding proteins *19* and *20*.

The results obtained in this study with *1003* cells may be compared with those obtained previously on *F9* cells [9]. Both cell lines have a similar

pattern of major surface proteins; nevertheless, main differences may be found in the medium-exposed surface proteins, as well as in their affinity for concanavalin A. In both *1003* and *F9* cells, the main concanavalin A-binding proteins are exposed to the culture medium, although in the differentiated cell lines studied (*10035, 10031* and *1168*) they become available for ^{125}I-labelling exclusively after the cell detachment.

A number of previously reported data outlined the rôle of concanavalin A-binding glycoproteins in cell adhesion, migration and differentiation. For example, a redistribution of concanavalin A-binding proteins occurs on the cell surface during myoblast differentiation [21]. A concanavalin A-binding glycoprotein isolated from embryonal carcinoma *F9* cells was suggested to be involved in the mechanism of morula compaction in the mouse embryo [22]. It was also shown that a redistribution of concanavalin A-binding compounds takes place during cell-cell interactions in early mouse embryo and that polarization plays an important role in embryonic differentiation [23–25].

All these data suggest that the group of concanavalin A-binding proteins might be involved into the mechanism of cell adhesion indispensible to cell differentiation.

In conclusion, results obtained in this work show that after different pathways of mesenchymal differentiation, the most significant changes occur in the group of cell-surface proteins exposed to the culture medium. Furthermore, our results have shown that the changes in the accessibility for iodination of concanavalin A-binding proteins take place after mesenchymal differentiation of embryonal carcinoma *1003* cells.

Acknowledgements

We wish to thank Professor F. Jacob for the interest in this work, Dr. D. Louvard for reading of the manuscript and critical comments, Dr. J. Gaillard for histological analysis of tumors and Mr L.K. Bagilet for his help with electrophoretic analyses. This work was in part supported by Fondation pour la Recherche Medicale Française.

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