Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 3747–3750, June 1984
Cell Biology

Invasive behavior of mouse sarcoma cells is inhibited by blocking a 37,000-dalton plasma membrane glycoprotein with Fab fragments

(cancer/cell invasiveness/cell surface antigens/monoclonal antibodies)

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Communicated by A. Frey-Wyssling, February 14, 1984

ABSTRACT **Abercrombie's confronted explant technique was used to study the role of tumor surface antigens in malignant invasion. Plasma membranes were isolated from mouse sarcoma cells (FS9) and a mouse cell line (L929) of the same *H-2* haplotype. FS9 cells are highly invasive when confronted with chicken heart fibroblasts, whereas the L929 cells are not [Abercrombie, M. (1979) *Nature (London)* 281, 259–262]. The FS9 plasma membranes contained significantly higher concentrations of a 37,000-dalton glycoprotein. When antiserum directed against FS9 plasma membranes was preabsorbed with L929 cells, the antibodies remaining reacted predominantly with the 37,000-dalton antigen. Fab fragment prepared from the preabsorbed antiserum inhibited the invasion of chicken heart fibroblasts by FS9 cells. Fab prepared from a monoclonal antibody directed against the 37,000-dalton antigen also inhibited invasivity, whereas monoclonal antibodies reacting with two other FS9 cell surface antigens did not. The results imply a relationship between the increased concentration of the 37,000-dalton glycoprotein on the surface of the FS9 cells and their invasivity.**

The surfaces of tumor and normal cells are believed to differ in one or more significant aspects (1). Metastasis involves detachment of cells from the primary tumor, their adhesion to vascular endothelia at distant sites, invasion of the vessel wall, and growth in the stroma (1, 2). The ability of metastatic cells to participate in cell–cell and cell–endothelial matrix interactions may be associated with their invasive properties. Hence, tumor cell-surface proteins may mediate metastasis and, in fact, fusion of membrane vesicles can transfer the potential for a higher rate of metastasis to poorly metastatic sublines (3). However, attempts to detect qualitative differences in surface properties between cells with different metastatic potential have been inconclusive (1–7). Studies using mouse B16 melanoma metastatic variants suggested that differences in surface proteins may be related to greater protease or glycosidase activity in cells with lower metastatic potential (8). Recently, some of the monoclonal antibodies that blocked adhesion of B16 cells to tissue culture dishes were also found to reduce formation of metastases *in vivo* (9). The antibodies were directed against antigens on the surface of B16 but not normal mouse cells.

We chose to use the Abercrombie confronted explant technique to study the role of surface proteins in the invasive behavior of malignant cells (10–13). In this technique an explant of malignant cells is confronted with a standardized chicken heart explant. The mouse sarcoma (FS9) cells we selected to study show nonreciprocal invasion (13). When the outgrowths of the two explants meet, the chicken heart fibroblasts are almost totally obstructed, whereas the outward drive of the FS9 explant persists into the fibroblast explant's zone. The mouse cell line L929 was selected as a control because these cells show a high degree of obstruction when confronted with chicken heart fibroblasts and have the same *H-2* haplotype as FS9 cells. We initially looked for differences in the plasma membrane proteins of the two mouse cell lines. We then isolated antibodies that were specifically directed against either FS9 or L929 plasma membranes and determined whether the Fab fragments could influence the behavior of the cells in the confronted explant test. Our results suggest that blocking a specific antigen on the surface of FS9 cells with Fab can inhibit their invasivity.

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MATERIALS AND METHODS

Cell Cultures. L929 and FS9 cells were grown *in vivo* in CBA mice and taken in culture in Falcon 3024 flasks in Dulbecco-modified Eagle's medium with 10% fetal bovine serum (KC Biological, Lenexa, KS), penicillin at 100 units/ml, and streptomycin at 100 μg/ml, bicarbonate-buffered (pH 7.2) in 5% CO_2 in air (moist atmosphere).

Plasma Membranes. Plasma membranes were isolated by using the Tris·HCl method of Atkinson and Summers (14) modified according to Hubbard and Cohn (15) from cells that had been cultured for 5 days after the previous passage. Approximately 2 mg of membrane protein was isolated from 4 × 10^8 cells. Membrane proteins were separated on Na-DodSO$_4$/10% acrylamide gels according to Laemmli (16). Proteins were detected by silver staining of gels (17) and a modified silver stain specific for glycoproteins was also used (18).

Immunization. Rabbits were injected intramuscularly with 2 mg of membrane protein in 1 ml of phosphate-buffered saline mixed with 1 ml of Freund's complete adjuvant. Four booster injections, using Freund's incomplete adjuvant, were given at 4- to 6-week intervals. Blood was collected 10 days after the last booster injection, serum was heated at 56°C for 30 min, and possible anti-fetal calf serum antibodies were removed by affinity chromatography (19). Activity of the antiserum was checked by indirect immunofluorescence. Fab fragments were prepared according to Porter (20).

Antigen Detection. Membrane proteins separated on Na-DodSO$_4$ gels (see Fig. 1) were transferred electrophoretically to nitrocellulose filters (Schleicher & Schüll, BA 85, 0.45-μm pore diameter) (21) in 25 mM sodium phosphate buffer, pH 7.0. Antigens were detected by incubating the filters with monoclonal antibody (purified on DEAE-cellulose; DE-52, Whatman) at 80 μg/ml or polyclonal antisera diluted 1:25 in incubation buffer (15 mM sodium phosphate, pH 7.2/0.9% NaCl/1% bovine serum albumin) for 2–3 hr at room temperature or overnight at 4°C. Filters were washed three times, 15 min each, in 15 mM sodium phosphate buffer, pH 7.2/0.9% NaCl and peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse IgG (Miles-Yeda, Rehovot, Israel; diluted 1:200) added in incubation buffer. After incubation for 60 min fil-

ters were washed three times and stained for 10 min with 50 mg of 3',3-diaminobenzidine·4HCl (Fluka) in 100 ml of 100 mM sodium phosphate buffer (pH 7.2) containing 20 μl of H_2O_2 (30%, wt/vol). The filters were washed in buffer and dried.

Preabsorbed Antisera. Preabsorbed antisera were obtained by incubating 1 ml of anti-FS9 or anti-L929 antiserum with 7 × 10^7 L929 or FS9 cells, respectively. Cells were washed previously with phosphate-buffered saline lacking Mg^{2+} and Ca^{2+}. Incubation was for 60 min at 4°C in a roller culture (34 rpm, Multipurpose Rotator, Scientific Industries, Springfield, MA). Cells were removed by centrifugation and the supernate was reincubated with fresh cells a further five times. The final supernate was centrifuged at 15,000 rpm for 10 min.

Confronted Explant Test. Ethanol-washed coverslips were coated with a uniform film of collagen (Vitrogen 100, Flow Laboratories), dried in sterile air, and washed with phosphate-buffered saline prior to use (10, 22). Explants (0.5 mm^3) from FS9 tumors grown *in vivo* or from 9- to 10-day-old chicken embryo heart ventricle were placed 0.8–1.2 mm apart on the coverslips. Clumps of FS9 cells grown in culture could also be used in place of tumor explants. The coverslip cultures were incubated in Dulbecco-modified Eagle's medium containing 10% (vol/vol) fetal calf serum in an atmosphere of 5% CO_2 in air; medium was renewed daily. The cultures were fixed and Giemsa stained 12–24 hr after cells from the two explants had met.

Monoclonal Antibodies. Mice were immunized with FS9 plasma membranes and fusion of the BALB/c and FO cells was carried out 3 days after the fourth booster had been given. Monoclonal antibodies were isolated (23, 24) and tested in the staphylococcal protein A assay (25).

RESULTS

Comparison of Plasma Membrane Proteins in FS9 and L929 Cells. Plasma membranes were isolated from FS9 mouse sarcoma cells and L929 mouse cells, which have the same *H-2* haplotype as FS9 cells. Protein patterns on silver-stained $NaDodSO_4$ gels showed a number of differences between the two cell types (Fig. 1). Conspicuous was a 37,000-dalton glycoprotein that occurred at much higher concentrations in FS9 plasma membranes. This protein was labeled after galactose oxidase treatment and sodium boro[^{3}H]hydride reduction of whole cells (not shown) (26).

Antigenic Differences Between Plasma Membranes of FS9 and L929 Cells. Antibodies specifically directed against one or the other of the two cell types were obtained by preabsorption of the antisera with cells. After preabsorption of anti-FS9 plasma membrane antiserum with L929 cells, the preabsorbed antiserum reacted predominantly with only one antigen in the FS9 plasma membranes (Fig. 2*a*, lane i). This was the 37,000-dalton glycoprotein already identified on the silver-stained gels (Fig. 1). A number of weakly stained bands were detected, indicating antigens present in low concentrations or binding small amounts of antiserum. Variability in these weak bands between experiments suggests they may result from incomplete absorption of antiserum by L929 cells.

We tested whether the 37,000-dalton glycoprotein was a histocompatibility antigen by incubating the protein blots of FS9 plasma membranes with C57BL/6 anti-CBA antiserum. The antiserum bound to a 45,000-dalton protein (not shown).

When antiserum directed against L929 plasma membranes was preabsorbed with FS9 cells, it subsequently reacted predominantly with a 55,000-dalton protein of the L929 plasma membranes (Fig. 2*b*, lane ii). The nature of this protein is unclear. Some weak bands were again detected on the blots.

Effect of Fab Fragments on Invasiveness. We added Fab fragments derived from the preabsorbed sera to Abercrombie's confronted culture system and examined the effects on invasiveness. When two explants (foci of cells) of chicken heart and mouse L929 cells were placed about 1 mm apart, outwandering of the cells occurred, eventually leading to collision between the two cell types. After junction, both chicken and L929 mouse cell migration was obstructed (not shown) (10–13). However, when FS9 mouse cells were used, nonreciprocal invasiveness was observed (13). The chicken heart fibroblasts usually moved out of the way of the invading FS9 cells or were overrun (Fig. 3a). Obstruction can be

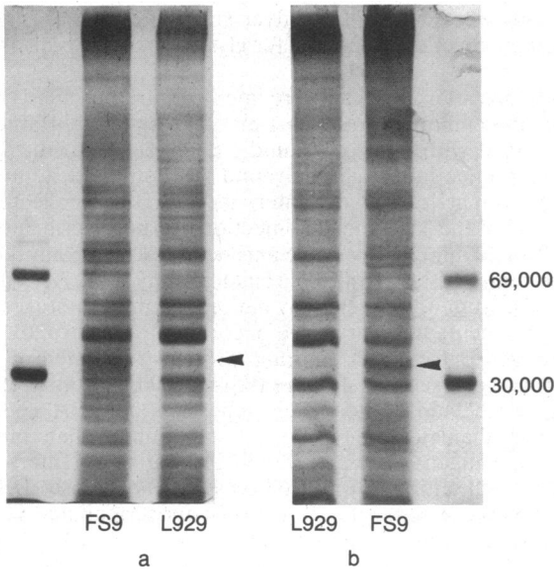

FIG. 1. Comparison of proteins in plasma membranes isolated from L929 and FS9 cells. (*a*) Gel stained with silver. (*b*) Modified silver stain to detect glycoproteins. The arrow indicates a 37,000-dalton glycoprotein found in significantly greater concentrations in the plasma membranes from FS9 cells.

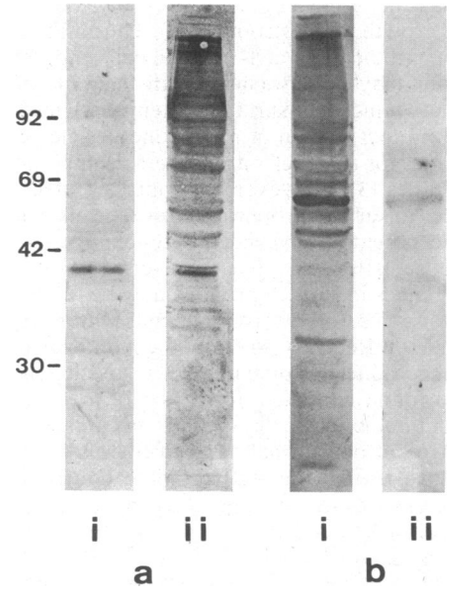

FIG. 2. Comparison of antigens in L929 and FS9 plasma membranes. (*a*) FS9 plasma membrane; filters incubated with antiserum directed against FS9 plasma membranes. Lane i, antiserum preabsorbed with L929 cells; lane ii, nonpreabsorbed antiserum. (*b*) L929 plasma membrane; filters incubated with anti-L929 plasma membrane antiserum. Lane i, nonpreabsorbed antiserum; lane ii, antiserum preabsorbed with FS9 cells.

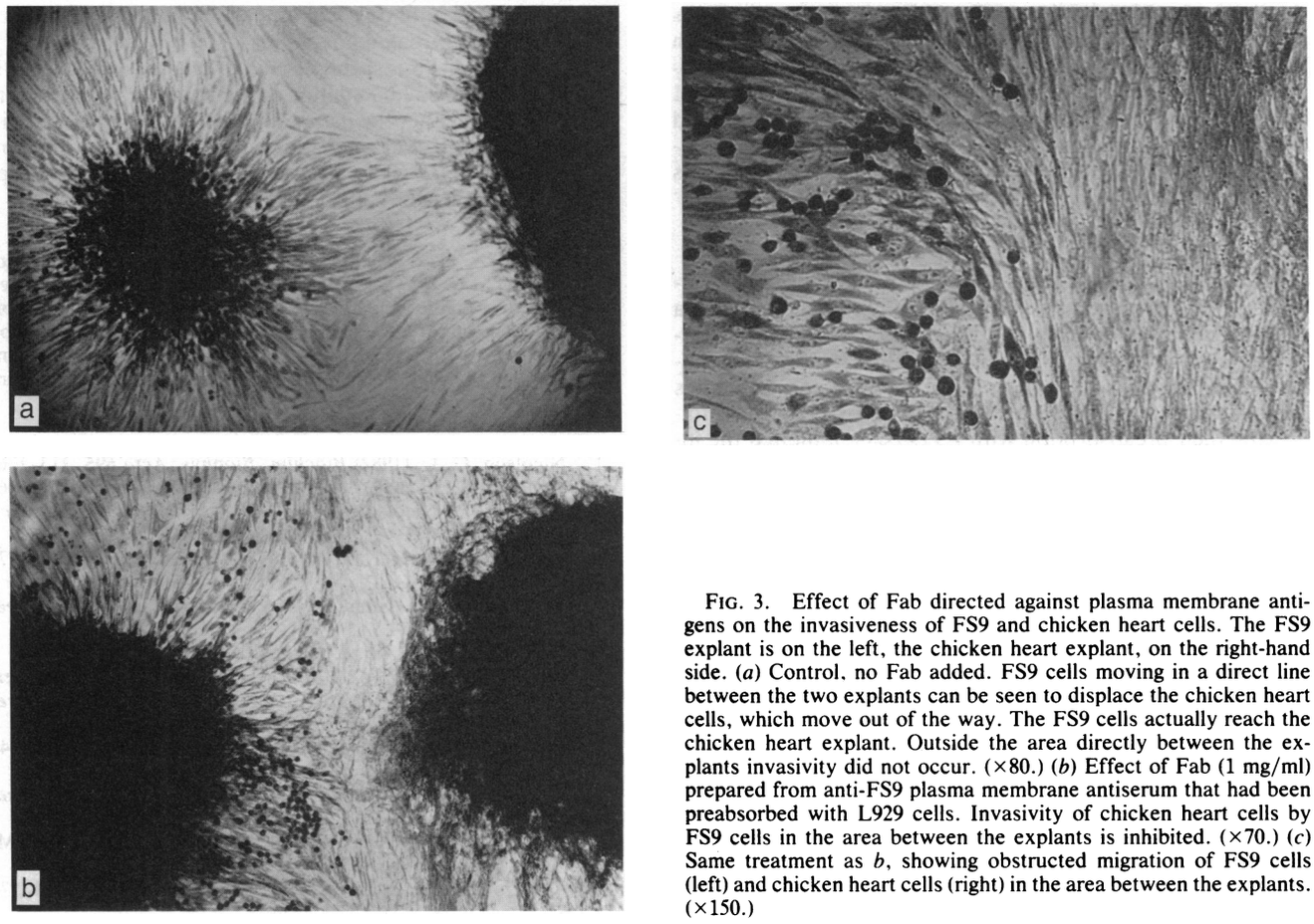

FIG. 3. Effect of Fab directed against plasma membrane antigens on the invasiveness of FS9 and chicken heart cells. The FS9 explant is on the left, the chicken heart explant, on the right-hand side. (*a*) Control, no Fab added. FS9 cells moving in a direct line between the two explants can be seen to displace the chicken heart cells, which move out of the way. The FS9 cells actually reach the chicken heart explant. Outside the area directly between the explants invasivity did not occur. (×80.) (*b*) Effect of Fab (1 mg/ml) prepared from anti-FS9 plasma membrane antiserum that had been preabsorbed with L929 cells. Invasivity of chicken heart cells by FS9 cells in the area between the explants is inhibited. (×70.) (*c*) Same treatment as *b*, showing obstructed migration of FS9 cells (left) and chicken heart cells (right) in the area between the explants. (×150.)

assessed by using the invasivity index, a measure of the distance of infiltration standardized for the outward velocity from the explant (12, 13). The invasivity index, which is 0 for a cell type totally stopped by the other and 1.0 for a cell type totally unimpeded, reached values above 0.9 for FS9 cells. [Abercrombie (13) reported values of 0.6 for FS9 cells and 0.1 for chicken fibroblasts.] At the sides of the explants, invasion by FS9 cells did not occur (Fig. 3*a*). This confirms results showing the angle of contact between cells is important in determining invasivity (27–29). Hence, the relevant surface molecules may be concentrated at the front of the cell.

When Fab derived from anti-FS9 plasma membrane antiserum preabsorbed with L929 cells was added to the culture, FS9 invasivity was completely inhibited (Fig. 3 *b* and *c*). The invasivity index for both cell types was never more than 0.1. This effect was observed at Fab concentrations of 0.2 mg/ml and above. When Fab derived from control IgG or from anti-L929 plasma membrane antiserum preabsorbed with FS9 cells was used, no effect on FS9 cell invasivity was observed (not shown). Fab from nonpreabsorbed antisera could not be used as it resulted in release of cells from the substrate, possibly due to the presence of antibodies directed against fibronectin (9).

Monoclonal Antibodies. We isolated a number of monoclonal antibodies directed against FS9 plasma membrane antigens. Monoclonal antibodies DD9 and DG9 bound specifically to FS9 cells, whereas A4 was bound in equal amounts by FS9 and L929 cells. Immunoblotting indicated that DD9 bound to the 37,000-dalton antigen and A4 to a 13,000-dalton antigen (Fig. 4). However, DG9 failed to bind to any proteins on the nitrocellulose filter (Fig. 4). (This was the case with two-thirds of the monoclonal antibodies tested, presumably due to poor transfer or blocking of antigenic sites when proteins were bound to the filter.)

Fab fragments were prepared from the monoclonal anti-

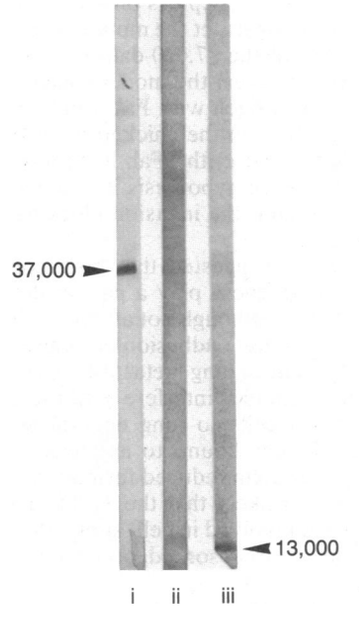

FIG. 4. Identification of antigens reacting with monoclonal antibodies directed against FS9 plasma membrane. FS9 plasma membrane proteins separated on $NaDodSO_4$ gels were electroblotted and the filters were incubated with monoclonal antibodies DD9 (lane i), DG9 (lane ii), and A4 (lane iii). Numbers on the left and right represent molecular size in daltons.

bodies and added to the confronted culture system. Fabs from A4 and DG9 added at up to 1 mg/ml had no effect on the invasivity of FS9 cells. However, Fab from DD9 completely inhibited FS9 invasivity at concentrations as low as 1 μg/ml, giving results identical to those shown in Fig. 3 *b* and *c*. Binding of DD9 Fab to FS9 cells was tested by using immunofluorescence and the protein A assay. Concentrations down to 1 ng/ml were positive; 0.1 ng/ml was negative.

DISCUSSION

$NaDodSO_4$ gel electrophoresis showed that some differences were present in the protein composition of plasma membranes isolated from L929 and FS9 cells. These differences were apparently related to the different invasive behavior of the two cell types, since monovalent antibodies (Fab fragments) directed against FS9 cell membranes, following their preabsorption with L929 cells, inhibited the invasivity of FS9 cells in the confronted explant test. Conversely, antibodies against L929 cell membranes, which had been preabsorbed with FS9 cells, did not affect the invasive behavior of either mouse cell type.

A 37,000-dalton cell surface glycoprotein appeared to be the antigen most likely involved in the inhibitory effect of Fab on FS9 cell invasion. This antigen bound over 90% of the antibodies in the preabsorbed antiserum that reacted with FS9 plasma membrane proteins. This interpretation was confirmed by showing that Fab prepared from a monoclonal antibody directed against the 37,000-dalton antigen also completely inhibited FS9 cell invasion. Fabs from two other monoclonal antibodies that reacted with FS9 plasma membrane antigens had no effect on invasivity even at high concentrations. The 37,000-dalton antigen is also detectable in relatively low amounts on L929 cells. FS9 cells bind 15 times more of the monoclonal antibody (DD9) than do L929 cells.

A monospecific antiserum to the gp37 molecule of Rous sarcoma virus (provided by E. Hunter) did not react with the 37,000-dalton antigen of FS9 plasma membranes.

The mechanism by which the 37,000-dalton antigen affects invasivity is unknown. The simplest model is that blocking of the glycoprotein by receptors on the chicken heart fibroblasts is required to obstruct the mouse cells. Since FS9 cells possess an excess of the 37,000-dalton antigen, no obstruction can occur. However, the model appears unsatisfactory since blocking the antigen with Fab would presumably also prevent the receptors on the chicken heart fibroblasts from binding and, furthermore, the Fab itself might simulate cell contact. An alternative hypothesis is that excess 37,000-dalton antigen can inhibit the invasion-blocking mechanism of the fibroblasts.

Abercrombie has suggested that both contact inhibition and variable adhesiveness play a part in determining invasiveness (13). Some, although not all, monoclonal antibodies that inhibit cell–substrate adhesion of mouse B16 melanoma cells *in vitro* also reduce lung metastasis *in vivo* (9). This may be because the antibodies interfere with the adhesion of circulating melanoma cells to lung endothelia. Three of the monoclonal antibodies bound to antigens of 40,000-50,000 daltons, and two of them reduced formation of lung colonies *in vivo* (9). It is not likely that the 37,000-dalton antigen of FS9 mouse cells is involved in cell–substrate adhesion, since neither addition of preabsorbed polyclonal antibodies nor the monoclonal antibody had any effect on the adhesion of cells to collagen-treated coverslips.

It is important to test the *in vivo* effects of antibodies directed against the 37,000-dalton antigen. It will also be interesting to see whether the increased levels of the glycoprotein in tumor cells are due to enhanced transcription, possibly by amplification of the gene, and whether the gene has the properties of an oncogene (30).

We thank Dr. G. A. Dunn (Medical Research Council Cell Biophysics Unit, London) for helping us to introduce the confronted culture system and for supplying FS9 cells. We are grateful to Prof. Jean Lindenmann for his support. The work was financed in part by the Hartmann–Müller-Stiftung, Swiss Cancer Society Grant FOR.203.AK.81, and Swiss National Science Foundation Grant 3.222.-0.82.

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