DISTRIBUTION OF FIBROBLAST SURFACE ANTIGEN
Association with Fibrillar Structures of Normal Cells and Loss Upon Viral Transformation*

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Much study is currently directed towards understanding the role of cell surface components in maintenance of the normal differentiated phenotype and in expression of the altered growth properties of malignant cells (1, 2). Using an immunochemical approach we recently described in chick embryo fibroblasts a surface antigen (3) that is decreased or absent after malignant transformation by Rous sarcoma virus (RSV) (4). This component, tentatively designated as SF antigen (SFA), has several additional properties that warrant a detailed analysis of its expression. It is a major surface antigen identifiable as distinct high molecular weight polypeptides one of which has a uniquely high rate of turnover (4, 5). It is cell type-specific and is exposed at the surface of fibroblasts but not on other cell types studied. SFA is also present in homologous serum (3, 6).

Preliminary findings suggested that the antigen is unevenly distributed on the surface of cultured chick fibroblasts (3). In order to investigate the localization of the antigen a method was adopted in which immunofluorescence could be correlated with scanning electron microscopy of the same cells.

In this report we demonstrate that SFA is confined to membrane processes and surface ridges of normal fibroblasts. Conventional trypsinization used to subculture cells removes all detectable SFA (4). This has permitted us to study the reappearance of SFA and cell surface structures when cells after reseeding attach, spread, and regain their normal differentiated fibroblast phenotype. The cell surface antigen is also lost during transformation of fibroblasts to the malignant phenotype and reappears in the reverse process, detransformation.

Material and Methods

Cells. Fibroblasts from the body walls of 10-day old c/o Brown Leghorn chick embryos were prepared and grown as described previously (7). Secondary cultures were grown at +39°C in medium 199 supplemented with 10% tryptose phosphate broth and 5% calf serum. They were trypsinized

1Abbreviations used in this paper: PBS, phosphate-buffered saline; RSV, Rous sarcoma virus; SFA, soluble fibroblast surface antigen.

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according to conventional procedures (0.05% trypsin, 15 min, +39°C) and seeded, 5 x 10⁶ cells per 20 cm², to dishes containing small carbon-covered slips marked with diamond drawn grooves. The cultures were used within 1-2 days.

Transformation. The origin of the avian type C viruses used has been given elsewhere (4). RAV-1 is a nontransforming avian leukemia virus, RSV-SR-A is RSV Schmidt-Ruppin strain subgroup A, and RSV(RAV-1) a Bryan strain of RSV. RST-Ts-68 is an RSV mutant temperature-sensitive for transformation (8). Fibroblasts infected with this mutant assumed a normal phenotype within 12-24 h at +41°C but were transformed at +36°C. Secondary chick embryo fibroblasts infected with RSV as described earlier (4), were grown in medium 199 supplemented with 10% tryptose phosphate broth and 5% calf serum. Unless otherwise indicated the transformed cells were subcultured once before use in the experiments. The medium was changed 16-20 h before harvesting of the cultures for immunofluorescence and scanning electron microscopy.

Anti-SF Antibodies and Immunofluorescence. Preparation of antichicken SF sheep antisera has been described earlier (3). A digest of extensively washed live chick fibroblast cultures was obtained using insolubilized papain, clarified by centrifugations, dialyzed, lyophilized, and used to immunize a sheep. The antiserum was absorbed with calf serum (a component of the fibroblast growth medium).

Affinity purification of antichicken SF antibodies was achieved by absorption onto and elution from Sepharose particles to which chicken serum (that also contains chicken SFA) had been linked by the cyanogen bromide method (9). Staining of fibroblasts by anti-SF antibodies was specific: immunofluorescence of various chick embryo tissues, including liver, spleen, kidney, and muscle showed fluorescence only in loose connective tissue and within blood vessels. Addition of chicken serum, but not of calf or human serum, in the staining reaction blocked the fluorescence of fibroblasts.

For immunofluorescence the fibroblast cultures on cover slips were washed at room temperature once with Hanks’ salt solution (5 ml, 3 min) and rinsed twice with phosphate-buffered saline (PBS). The cells were fixed for 30 min in acetic acid at +20°C or in 1.5% glutaraldehyde (0.1 M Na-cacodylate, pH 7.2, with 0.2 M sucrose) at room temperature. The fixation was terminated by three washes with cold PBS. The cultures were incubated for 30 min with affinity-purified anti-SF sheep antibodies, washed three times with PBS and incubated for 30 min with anti-sheep gammaglobulin antibodies conjugated with fluorescein isothiocyanate (FITC isomer I, Baltimore Biological Laboratories, Cockeysville, Md.; rabbit anti-sheep gammaglobulin, F/P molar ratio 2.5). The stained cover slips were then washed for 30 min in PBS and mounted in buffered glycerol (pH 9.0) on microscope slides for examination and photographing under a Wild microscope using a halogen lamp as light source and an RITC interference filter combination (Optisk Laboratorium A.T.V., Lyngby, Denmark).

Scanning and Transmission Electron Microscopy. After immunofluorescence studies the carbon coated cover slips were gently detached from microscope slides by incubation in PBS. The cultures were dehydrated with the critical point method (10) using ethanol, amylacetate, and liquid CO₂ substitution. The dried specimens were covered with a layer of gold in a Balzers Micro BA 3 evaporator and examined with a Jeol SEM-U3 scanning electron microscope using 20 kV accelerating voltage and 40° specimen tilt.

For transmission electron microscopy the cells on the carbon layer were fixed with 1.5% OsO₄ in 0.1 M phosphate buffer, pH 7.2, for 1 h. The specimens were dehydrated in ethanol and embedded in Epon 812. Thin sections were double stained with uranyl acetate and lead citrate and examined in a JEM 100-B type electron microscope at 80 kV accelerating voltage.

Results

Distribution of SF Antigen in Fibroblasts. The immunofluorescence of SFA had a streaklike pattern on cultured chicken fibroblasts (Fig. 1). Large parts of the cell surfaces seemed to lack detectable antigen.

Reappearance of SF Antigen after Trypsinization. After trypsin treatment and seeding of cells SFA appeared first at the periphery of the rounded cells, at the sites of the first new cell processes (Fig. 2). The rate of appearance of SFA...
Fig. 1. Cultured chicken fibroblasts stained after acetone fixation with fluorescent antibodies against fibroblast SFA. In the two cells visible (A and B) the fluorescence has an uneven distribution and forms streaks oriented largely parallel to the long axes of the cells. × 1,500.

Fig. 2. Immunofluorescence pattern of SFA at 1 h after reseeding trypsin-treated fibroblasts. Acetone fixation. Some cells are negative, some show fluorescent staining at the cell periphery where developing cell processes have appeared. × 400.

Fig. 3. Acetone-fixed cell with more advanced SFA staining pattern and elongated morphology at 2 h after seeding. Insert: In addition to granular fluorescence also a strandlike distribution pattern of SFA is evident. The antigen-containing strands are within the cytoplasmic area but extend also out from the periphery of the cell. × 1,500. In SEM the cell has a disrupted surface with discreet ca. 50-100 nm wide ridges and peripheral processes, both of which correspond to fluorescent strands (arrows). At places small surface aggregates are located above the ridges and correspond to distinct fluorescent granules (arrows). × 30,000.
immunofluorescence was studied by two fixation methods, acetone and glutaraldehyde (Table I). With acetone fixation SFA was first detected after 1 h of cultivation. The staining of the cultures increased and reached its maximum after 4-10 h. With glutaraldehyde fixation the SFA was first detected at 10 h of culture and reached its maximum within 22 h. The intensity of the staining glutaraldehyde-fixed cells remained weaker than in acetone-fixed cultures.

Comparison of Immunofluorescence with Scanning Electron Microscopy (SEM). The surface of cells fixed in acetone appeared in SEM grossly damaged with excavations and a network of irregular structures (Fig. 3), whereas glutaraldehyde-fixed cells had smooth and well preserved surface structure (Figs. 5, 6). The immunofluorescent staining after acetone fixation (Fig. 3 insert) correlated in SEM with cell surface ridges and peripheral membrane processes both 50-100 nm in diameter (Fig. 3).

| Time after seeding (h) | Acetone | Glutaraldehyde |
|-----------------------|---------|----------------|
| 0.5                   | -       | -              |
| 1                     | +       | -              |
| 2                     | ++      | -              |
| 4                     | ++      | -              |
| 10                    | +++     | +              |
| 22                    | +++     | ++             |

The intensity of the specific immunofluorescence was rated from - to +++.

Glutaraldehyde-fixed cells had in SEM numerous membrane processes that corresponded to the SFA-containing streaks in immunofluorescence (Figs. 4, 5). The diameter of the SFA-containing fibrillar structures varied from 50 to 200 nm. The processes connected the cells to the substratum, and to the neighbouring cells extending frequently long distances on the cell surfaces (Fig. 5).

Some cells had especially numerous fluorescent streaks over the cell body. In such cells the immunofluorescence pattern (Fig. 6 insert) corresponded to discreet surface ridges seen in SEM (Fig. 6).

The immunofluorescence pattern and structures seen in SEM correlated in general well. Lack of complete agreement in the photographs appeared to result from the limited sensitivity of the photographic procedure, the fact that only part of the fluorescent structures seen in the microscope were in focus at one time, and the fragmentation of thin membrane processes taking place during preparation for SEM.
FIG. 4. Immunofluorescent staining for SFA of glutaraldehyde-fixed cells at 10 h after seeding. The specific fluorescence is strong and has a strandlike pattern. $\times$ 750.

FIG. 5. SEM of cells seen in Fig. 4 shows that the cells have achieved a fibroblast-like phenotype with numerous membrane processes attaching them to substratum or reaching from cell to cell. The fibrillar membrane processes correspond largely to the SFA-containing streaks in Fig. 4 (arrows). $\times$ 4,500.

FIG. 6. Glutaraldehyde-fixed fibroblast at 22 h after seeding. Insert: Fluorescence shows SFA as streaks over the cell body. In this case also a radial pattern with a central halo was present. $\times$ 750. In SEM the fluorescent streaks of the insert are seen to correspond to cell surface ridges in great detail (arrows). $\times$ 4,500.
Thin Sections of the Cytoplasmic Processes. Since SFA was localized to the cytoplasmic processes (Figs. 3–5), 50–200 nm in diameter, it was of interest to study their ultrastructure. Thin sections of glutaraldehyde-fixed preparations demonstrated that the cellular processes limited by a distinct plasma membrane contained microtubules and bundles of microfilament-like structures with a diameter between 5 to 10 nm. These were best seen in longitudinal sections of the cells (Fig. 7). In acetone-fixed preparations the cell surface membrane was destroyed and the microtubules and microfilament-like structures were not visible (Fig. 8).

**Fig. 7.** Transmission electron micrograph of a fibroblast fixed with glutaraldehyde 22 h after trypsinization and seeding, the cell process, limited by a distinct surface membrane contains both microtubules (MT) and microfilament-like structures (MF). × 90,000.

**Fig. 8.** Transmission electron micrograph of acetone-fixed fibroblast at 22 h after subculture. The cell surface is grossly disrupted. No distinct surface membrane is visible and the cytoplasm contains aggregated material and cavities. × 90,000.

Disappearance of SFA in Transformed Cells. The majority (>90%) of individual RSV-transformed fibroblasts were found to have no detectable antigen in immunofluorescence. A small proportion of the cells with the transformed phenotype had localized areas containing SFA (Fig. 9). This spotlike antigen distribution was distinctly different from the fibrillar pattern typical of untransformed control fibroblasts (Fig. 10). The Schmidt-Ruppin and Bryan strains of RSV gave similar results. Fibroblasts infected with RAV-1, a nontransforming leukosis virus, showed the same pattern of SFA distribution as uninfected fibroblasts.
Extracellular Antigen in Transformed and Normal Cultures. In the above experiments the fibroblasts had been transformed with RSV and then subcultured once before immunofluorescent staining. Under those conditions little if any extracellular antigen was seen. If, however, the cells had not been subcultured after transformation considerable amounts of extracellular antigen could be detected as a fibrillar network under the cells (Fig. 11). A similar SFA-containing network was seen under normal chick fibroblasts between the growth substrate and the cells after several days of cultivation (Fig. 12). The two layers, the surface layer and the extracellular layer underneath, could be readily distinguished by focusing the microscope. The extracellular layer was particularly evident in dense old cultures.

SFA in Fibroblasts Temperature Sensitive for Transformation. Fibroblasts infected with RSV-Ts-68, a virus mutant temperature-sensitive for transformation, showed a transformed phenotype as regards the presence of SFA when cultured at +36°C (Fig. 13) and a normal phenotype when cultured at +41°C (Fig. 14). A small proportion of the infected cells retained a normal fibroblastic morphology when maintained at the permissive temperature (+36°C). These cells also had an SFA pattern typical of normal fibroblasts.

In shift-up experiments (+36°C → +41°C) SFA, as detected by immunofluorescent staining of acetone-fixed cultures, reappeared within 1-2 h of incubation at +41°C. The new antigen was confined to areas of the reappearing fibroblastic extensions (Fig. 15). In glutaraldehyde-fixed cultures the first new antigen was seen at about 6 h after the shift-up of the incubation temperature. Antigen
quantity in shift-down (+41°C → +36°C) transformation experiments was difficult to evaluate as considerable amounts of antigen remained trapped in the extracellular space under the cells. If cells maintained at +41°C were trypsinized and subcultivated at +36°C the cells assumed the transformed morphological phenotype with no or little antigen.

Discussion

These studies demonstrate that SFA, a major cell type-specific surface antigen, has a highly nonrandom distribution in fibroblasts. The pattern of immunofluorescence suggests that the antigen is associated with streaks that often have an orientation parallel to the cellular extensions. SEM correlated the antigen with cell surface ridges and peripheral membrane processes that frequently extended from the cell body to the intercellular and extracellular areas. Such ridges and membrane processes are characteristic to cultured fibroblasts (11-13).

The time of reappearance of detectable SFA during cell spreading was dependent upon the mode of fixation for immunofluorescence. The first new antigen appeared considerably earlier in the series of acetone-fixed than in glutaraldehyde-fixed cells. It seems that this is not simply due to cross-linking and inactivation of the antigen molecules by the latter fixative since extracellular SFA-containing structures stained well after glutaraldehyde fixation in immunofluorescence. A more likely explanation is that SFA is localized at different levels relative to the external cell surface and that an increased number of antigen sites become accessible to antibodies after acetone fixation.

Trypsinized spherical cells have no detectable SFA either in immunological assays (4) or in immunofluorescence as shown here. During cell spreading SFA was first detected at the developing cellular extensions. Electron microscopy of sections has revealed bundles of both microtubules and microfilaments along cell processes of fibroblasts. Large bundles of microfilaments are known to be localized just under and in contact with the plasma membrane of fibroblasts and other cells, preferentially at the sites of contact with neighbouring cells and growth support (14-18). The SFA-containing streaks and the cell surface structures visible in SEM had similar distribution and dimensions. The membrane processes seemed in thin sections to contain bundles of microfilaments. Perdue (19) has also demonstrated such microfilaments in chicken fibroblasts.

Our data on the distribution of SFA are compatible with the possibility that the antigen is associated with microfilaments present in the membrane processes and under the surface ridges. This notion is made more likely by our observation (5) that a 45,000 polypeptide that comigrates with purified fibroblast actin (20) is associated with immunoprecipitates of fibroblasts SFA.

Whatever the relation between SFA and microfilaments, its uneven distribution implies anchoring to stable structures either outside or inside the cell surface to prevent free lateral movement of the antigen molecules (21–22). Fibrillar structures like microfilaments located within or underneath the cell membrane could provide such a fixation. Similar transmembrane mechanisms have recently been proposed to account for localized restraints on the mobilities of lectin receptors on the surface of sperm cells (23).
It may be significant that a decrease of cell surface SFA occurs besides in transformation also in other conditions with increased concanavalin A (Con A) effected agglutinability (2). Microgram quantities of trypsin known to make chick embryo fibroblasts agglutinable by Con A (24) and to initiate proliferation in density-inhibited cultures (25, 26) will release SFA from fibroblast monolayers into the medium. Surface of mitotic cells, also agglutinable with Con A, are devoid of SFA (unpublished observations). Anchorage of SFA in normal fibroblasts to filamentous structures could be a critical restraint to membrane mobility. Its lack could result in increased structural instability of the cell surface expressed as Con A agglutinability.

The present results show that fibroblasts after transformation by RSV contain little or no detectable SFA in immunofluorescence. This is consistent with our earlier immunochemical assays which have indicated that transformed fibroblasts have about 5-20% of SFA levels of control cultures, and the observation that extracts of transformed cells in SDS-gel electrophoresis lack the SF polypeptides (4). The 5-20% background seems to come from the following three sources: (a) a few untransformed cells are always seen; (b) a minority of transformed cells have aggregates of SFA containing fibrillar structures; and (c) SFA-containing structures may have become adhered to the substratum before or during the transformation process.

The cellular SFA complex is composed of three polypeptides with mol wt of about 210,000, 145,000, and 45,000 (5). The loss of SFA upon transformation of fibroblasts (4) may explain the recent observations by several groups that polypeptides of similar sizes are missing in the gel electrophoresis pattern of transformed fibroblasts or their membrane preparations from several species (27-32). These data and our unpublished observation (Vaheri and Ruoslahti, manuscript in preparation) that the human counterpart of SFA (6) is lost from the cell surface of SV40-transformed human fibroblasts suggest that disappearance of a high molecular weight cell surface protein is a general phenomenon associated with transformation of fibroblasts.

Summary

The localization of a cell type-specific, soluble fibroblast surface antigen (SFA) was studied by immunofluorescence and by scanning electron microscopy of the same cells. The antigen had an uneven distribution forming streaks on chick embryo fibroblasts. It was localized to membrane processes and ridges, with a diameter of 50-200 nm. The processes extended from the periphery of the cells to the substratum or to other cells. Trypsin treatment completely removed detectable amounts of SFA. The antigen was detectable within 1 h after trypsin-treated cells were reseeded. The reappearance of SFA correlated with the restoration of membrane processes.

Fibroblasts transformed by Rous sarcoma virus (RSV) showed loss of all or most SFA. When normal cells were transformed without subcultivation and trypsinization a fibrillar extracellular network of SFA remained under the transformed fibroblasts while the cells themselves were negative in immunofluorescence. When fibroblasts infected by RSV mutants were transferred to nonpermissive temperature for transformation new SFA was detected within 2 h.
These data lead us to propose that loss of stabilizing and anchoring effect of SFA molecules in fibrillar cell surface structures may be critical in altered growth control and malignant transformation.

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