CELLULAR TUMORIGENICITY IN NUDE MICE

Test of Associations Among Loss of Cell-Surface Fibronectin, Anchorage Independence, and Tumor-Forming Ability

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ABSTRACT

Fibronectin (FN; also called large external transformation-sensitive [LETS] protein or cell-surface protein [CSP]) is a large cell-surface glycoprotein that is frequently observed to be either absent or greatly reduced on the surfaces of malignant cells grown in vitro. Because FN may be a useful molecular marker of cellular malignancy, we have carried out an extensive screening to test the specific association among the degree of expression of FN, anchorage-independent growth, and tumorigenicity in the athymic nude mouse. A variety of diploid cell strains and established cell lines were tested for the expression of surface FN by indirect immunofluorescence using rabbit antisera against human cold insoluble globulin, rodent plasma FN, or chicken cell-surface FN. Concomitantly, the cells were assayed for tumor formation in nude mice and for the ability to form colonies in methylcellulose. Tumorigenic cells often showed very low surface fluorescence, confirming earlier reports. However, many highly tumorigenic fibroblast lines from several species stained strongly with all three antisera. In contrast, the anchorage-independent phenotype was nearly always associated with tumorigenicity in ~35 cell lines examined in this study. In another series of expriments, FN-positive but anchorage-independent cells were grown as tumors in nude mice and then reintroduced into culture. In five of the six tumor-derived cell lines, cell-surface FN was not significantly reduced; one such cell line showed very little surface FN.

Our data thus indicate that the loss of cell-surface FN is not a necessary step in the process of malignant transformation and that the growth of FN-positive cells as tumors does not require a prior selection in vivo for FN-negative subpopulations.

KEY WORDS fibronectin · cell transformation · anchorage independence · tumorigenicity · nude mouse

Malignant transformation of animal cells involves changes in many cellular properties which can be studied in vitro. These generally include decreased sensitivity to factors which restrict the growth of normal cells, such as low serum concentration (8, 47), lack of a solid surface for anchorage (26, 52, 53), and contact inhibition (6, 57, 58). In addition to their greater cellular growth autonomy in vitro,

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transformed cells frequently differ from their non-malignant counterparts with respect to their cell morphology (43, 56, 58), membrane properties (such as lectin agglutinability [reviewed in reference 37], antigenic specificities [reviewed in reference 30], and transport of certain nutrients [21]), decreased organization of the intracellular actin-containing cytoskeletal components (34), increased secretion of proteolytic enzymes (31, 59), and lower intracellular concentrations of cyclic nucleotides (reviewed in reference 32). It is now known that many of these phenotypes expressed by transformed cells in vitro can be dissociated from their neoplastic growth potential in vivo. Several years ago, Freedman and Shin (11) and Shin et al. (46) demonstrated that anchorage independence, defined as the ability of cells to proliferate in a semisolid medium which prevents their attachment to the tissue culture plate (54), is the in vitro marker which is best correlated with the ability of cells to form tumors in nude mice. Since then, this correlation has been tested in a wide variety of systems and appears to have general validity (17, 22; reviewed in reference 12).

There are, however, a few known exceptions to the association between anchorage independence and tumorigenicity. Certain virus-transformed cells which have a high efficiency of plating in semisolid medium do not form progressively growing tumors in nude mice (reviewed in reference 51). In the case of SV40-transformed human fibroblasts, the cells are not actually rejected: instead, small, benign nodules containing viable cells form at the site of inoculation and persist for several weeks or months before eventually regressing (50; M. Greenberg and S. Shin, unpublished material). Mouse teratocarcinoma cells transformed by SV40 (SVTER cells) and then selected for anchorage-independent growth are nontumorigenic in syngeneic as well as in nude mice (S. Shin and W. Topp, unpublished material). However, several recent experiments suggest that the failure of some virus-transformed cells to form tumors in nude mice might be a result of an ability of these cells to stimulate a host immune response rather than to an inherently nonmalignant cellular phenotype. For example, highly malignant HeLa (human cervical carcinoma) cells which are persistently infected with certain viruses lose their ability to grow as tumors in nude mice (38). In addition, several laboratories have recently reported that nude mice have elevated levels of thymus-independent cytotoxic cells which can suppress the growth of certain

tumorigenic cells (reviewed in reference 15). Other classes of cells in which the anchorage-independent phenotype is not associated with tumorigenicity are those derived from certain epithelioid tissues, including hematopoietic stem cells (49), normal mouse macrophages (25), and normal mouse and rat embryonic liver and lung cells (V. Freedman and S. Shin, unpublished material).

However, at the present time we know of few exceptions to the statement that all transformed cells which are capable of neoplastic growth in vivo are anchorage-independent. The situation can therefore be summarized as follows: while a few anchorage-independent cells may be nontumorigenic in nude mice, tumorigenic cells are invariably anchorage-independent. These findings have led us to propose that the loss of the anchorage requirement in vitro is a necessary but not a sufficient correlate of cellular malignancy (12, 45).

In the last few years several other specific in vitro correlates of tumorigenicity have been proposed. One such proposal concerns the cell-surface glycoprotein identified independently by Hynes (18) and by Hogg (16) on the surfaces of certain nontransformed cell strains and cell lines but greatly reduced or absent in their virally transformed counterparts. Further analysis of this protein, subsequently designated the "large external transformation-sensitive (LETS)" protein by Hynes and Wyke (20), but also known as fibronectin (FN), cell-surface protein (CSP), cell attachment factor (CAF), and 250K protein, showed that its disappearance from the cell surface was correlated with malignant transformation in the systems examined (3, 13). These observations led to the suggestion that the absence of cell-surface FN might have general validity as an in vitro marker of cellular tumorigenicity. Other reports (7, 22, 33) indicated, however, that the correlation between these two properties does not extend to certain other cell systems.

The present study was undertaken to clarify several questions about the associations among anchorage independence, loss of cell-associated FN, and cellular malignancy in nude mice: (a) Are the loss of FN and tumorigenicity specifically correlated in a wide variety of cells? (b) Is the loss of FN directly related to the acquisition of the anchorage-independent phenotype? (c) Is the loss of both cell-associated FN and anchorage regulation of growth in vitro required for tumorigenicity in vivo? We have therefore analyzed a large number of permanent cell lines and diploid cell strains

from many species for the degree of association among these three parameters. The results reported in this paper indicate that tumorigenic cells frequently have low or undetectable quantities of cell-surface FN, but the loss of FN is not a specific in vitro marker for cellular malignancy.

MATERIALS AND METHODS

Cell Culture Conditions

All cells except for baby hamster kidney (BHK) 21 and its clonal derivatives were maintained in Dulbecco-Vogt-modified Eagle's medium supplemented with 10% fetal bovine serum. BHK 21 and clones derived from it were grown in McCoy's 5a medium plus 10% fetal bovine serum. Media and serum were purchased from the Grand Island Biological Co., Grand Island, N.Y. Cultures were tested periodically for mycoplasma contamination according to the method of Chen (5) and were consistently found to be negative.

Diploid Cell Strains

Mouse embryo fibroblasts (MEF) were prepared from 13- to 15-d whole mouse embryos as described by Pollack et al. (35). MT cells, a strain of normal human newborn fibroblasts, were prepared from fresh surgical biopsy material by Dr. Abraham S. Klein according to the standard procedure (14). Chick embryo fibroblasts (CEF) were prepared from minced tissues of 9- to 12-d whole chick embryos by Marian J. Jackson according to the method of Rubin (41). A strain of human embryo fibroblasts (HEF) was kindly provided by Dr. H. Klinger. A strain of normal rat fibroblasts (NRF) derived from a kidney explant was obtained from Dr. Dino Dina.

Cell Lines

WOR-6, a mutant of Chinese hamster lung fibroblasts, was selected for resistance to both ouabain and 8-azaguanine and has been described previously (11). LNSV, GM 638, and GM 639, human fibroblasts established in culture by transformation with SV40, were a gift from Dr. Carlo Croce; a fourth such line, SV80, was obtained from Dr. William Topp. The anchorage-independent clone NG-8 was derived from GM 638 following mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and subsequent selection in methylcellulose (M. Greenberg and S. Shin, unpublished material). The human cell line D98/AH2 is an 8-azaguanine-resistant mutant derived from HeLa (55). BHK 21, a spontaneously transformed line of Syrian hamster kidney cells, has been described (27); the clones BHK cl.1 and BHK cl.2, as well as a polyoma transformant (PySp1A) and a chemical transformant (DMN6A) were all derived from BHK 21 by Dr. Noël Bouck and Dr. Giampiero Di Mayorca, who generously provided them for this study. A subclone of the rat myoblast line L6 (61) was obtained from Dr. Bernardo Nadal-Ginard. Rat-1 is a line of rat

embryo fibroblasts previously called F2408 (29); 14B is an SV40-transformed derivative of rat-1 (2) and the two clones FL 1-4 and FL 3-8 are flat revertants of 14B (48). These cell lines were generously provided by Doctors Bettie Steinberg and Robert Pollack. The Syrian hamster embryo fibroblast cell line NIL-8 was obtained from Dr. Richard Hynes.

Determination of the Efficiency of Plating in Semisolid Medium

Between 10^3 and 10^6 cells were plated in culture medium containing 10% fetal bovine serum and 1.3% methylcellulose (Fisher Scientific Co., Pittsburgh, Pa., 4,000 cps) over a layer of 1.2% agar (Difco Bacto Agar, Difco Laboratories, Detroit, Mich.), as described by Risser and Pollack (39). The growth medium used was either McCoy's 5a (for BHK 21 and clones derived from BHK 21) or Dulbecco-Vogt-modified Eagle's medium (for all other cells). The efficiency of plating was determined after 14–21 d by counting all colonies >0.3 mm with the aid of a bacterial colony counter (Lab-Line Instruments, Inc., Melrose Park, Ill.).

Nude Mice and Test for Tumorigenicity

The nude mice used in these experiments were derived from a stock partially backcrossed to Balb/c. Care and maintenance of the colony and procedures for testing cellular tumorigenicity have been described previously (11, 44). Briefly, cells were trypsinized, resuspended in phosphate-buffered saline (PBS) at a concentration of 2 × 10^6 cells in 0.2 ml, and then 0.2 ml of this suspension was injected subcutaneously at a single site. Mice were monitored for tumor development for at least 4 mo after the injection.

Re-establishment of Cell Cultures from Tumors

This procedure has been described in detail elsewhere (10, 44). Briefly, a small piece of tumor tissue was surgically removed from the mouse, minced, and then passed through a fine stainless steel mesh. The resulting cell suspension, consisting mostly of single cells, was plated in regular culture medium. Vigorously growing cultures were almost always obtained after a few days.

Antisera

Rabbit antiserum against chick cell-surface FN was prepared by M. Jackson essentially by the technique of Yamada and Weston (64) and Yamada et al. (65), as follows. Early passage chick embryo cells were grown in glass roller bottles (Bellco Glass, Inc., Vineland, N.J.), extracted in serum-free medium containing 2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 M urea (Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y., ultrapure grade), and then precipitated with ammonium sulfate. The resulting precipitate was

resuspended in cyclohexylaminopropane sulfonic acid (CAPS) buffer, pH 11, and dialyzed against three changes of the same buffer. The preparation was reduced with 1.5 M β-mercaptoethanol and then analyzed by SDS polyacrylamide gel electrophoresis. The band containing the 250K protein was cut out of the gel, homogenized in an E-C Apparatus Cellector (E-C Apparatus Corp., St. Petersburg, Fla.), and an aliquot containing ~0.40 mg of 250K protein was injected into multiple sites in the foot pads and back of an adult rabbit. The rabbit was injected again 3 wk later with ~0.60 mg of the same protein preparation. 4 d later, serum was collected and tested for its staining activity in the indirect immunofluorescence assay described below.

Rabbit antiserum raised against the plasma FN of both mouse and rat and subsequently absorbed with bovine serum proteins was a generous gift of Dr. Erkki Ruoslahti. Monospecific rabbit antiserum against human cold insoluble globulin (CIG) was prepared by A. B. Chen and M. W. Mosesson as described elsewhere (3) and was a gift from Dr. Lan Bo Chen.

Test for FN by Indirect Immunofluorescence

Cells were grown to confluence on 12-mm glass coverslips (Bellco Glass, Inc., Vineland, N. J.), fixed in 2% paraformaldehyde, and then assayed as described by Chen et al. (3). Briefly, coverslips were washed extensively in PBS, layered with 10 μl of diluted antiserum, and incubated in a humidified box for 20 min at 37°C. They were then rinsed thoroughly in PBS and reacted with 10 μl of fluorescein isothiocyanate (FITC)-conjugated goat antiserum against rabbit IgG (Meloy Laboratories Inc., Springfield, Va.). After this second reaction, coverslips were again rinsed in PBS, mounted on microscope slides with Gelvatol (Monsanto Co., St. Louis, Mo.), and screened for fluorescence using a Zeiss Photomicroscope III with epi-illumination. Yamada (62) has demonstrated that cells treated in this manner are impermeable to antibody molecules and that therefore the fluorescent material detected by this procedure is located on or external to the cell surface.

RESULTS

Detection of Cell-Surface FN by Indirect Immunofluorescence

To visualize the cell-associated FN, cells were grown and fixed on coverslips and then assayed by indirect immunofluorescence as described by Chen et al. (3). In the present study, we used rabbit antisera directed against: (a) human cold insoluble globulin (abbreviated as anti-CIG); (b) chicken CSP (anti-CSP); or (c) rat and mouse plasma FN (anti-rodent FN). This last antiserum was pre-

absorbed against bovine serum proteins, resulting in the complete removal of cross-reactivity to bovine plasma FN but retaining reactivity to rodent FNs, as determined by Ouchterlony double diffusion test (E. Ruoslahti, personal communication). The fetal bovine serum used in the cell culture medium contains significant amounts of bovine plasma FN; certain cell types, including fibroblasts, produce significant amounts of collagen in vitro, and it is known that FNs bind to collagen with a high affinity (9, 42). It is therefore important to distinguish between the FN produced by the test cells and serum-derived bovine FN which may have become associated with the cell surface, either directly or by binding to the collagen matrix produced by the cells, during the cultivation of the cells. The inability of the anti-rodent FN antiserum to recognize bovine FN eliminates the possibility that the fluorescent material visualized in the indirect immunofluorescence assay is bovine plasma FN originating from the serum component of the culture medium.

Initially, FN assays were performed using the antiserum with the specificity which was most closely related to the species of the test cells. For example, human cells were tested with anti-CIG while hamster, mouse, and rat cells were tested with anti-rodent FN. Later on, test cells were assayed with each of the remaining two antisera. No significant differences were observed in the staining patterns produced by the three antisera for any of the cell lines tested, although the intensity of staining frequently varied in the expected direction. For example, normal human diploid fibroblasts (MT cells) showed a dense extracellular network of FN with all three antisera but the staining was more intense with anti-CIG than with either anti-CSP or anti-rodent FN.

These results indicate that the failure of a particular cell line to fluoresce after treatment with any one of the three antisera most likely reflects the genuine absence of FN from the cell surface rather than the inability of the antiserum to recognize a heterologous FN molecule. This confirms the validity of using antiserum raised against chick, human, or rodent FN to assay for the presence of FN in cells from other species, as we have done in this study. In addition, the observation that the removal of cross-reactivity to bovine plasma FN from the anti-rodent FN antiserum did not produce a detectable difference in the pattern of immunofluorescence in any of the cells we tested provides evidence that the FN detected

by the anti-CIG and anti-CSP antisera originates not from the fetal calf serum present in the culture medium but from the cells themselves.

Cell Surface Distribution of FN

In agreement with previous reports (3, 28), we found that the distribution of FN on the surface of highly FN-positive cells varied with both cell density and the length of time since the last subculture. In sparse cultures, small amounts of FN were present in the areas of cell-substratum contact. As the cultures approached confluence, thin fibers of FN began to appear over the surface of the entire cell and in the areas of cell-cell contact. Within a few days after reaching confluence, many types of cells exhibited an extensive extracellular matrix which completely obscured the outlines of the underlying cells. Careful focusing of the microscope revealed that this FN network was located primarily on top of the cells. In this study such cultures were scored as positive for "extracellular FN matrix." Alternatively, some types of FN-positive cells did not produce this dense matrix even when maintained at confluence for several days, but instead they showed staining over the cytoplasm and/or between cells and in the same plane as the cells; such cultures were scored as positive for "intercellular FN." Cultures which showed no staining above the very low background level seen with pre-immune serum (obtained from a rabbit which was not immunized against FN) were scored as negative. These three types of staining patterns are illustrated in Fig. 1. To minimize the effects of cell density and time in culture on the staining patterns observed, all the cultures were grown to confluence and fixed 2 d later to assay for FN.

## Correlation Between Anchorage Independence, Loss of FN, and Tumorigenicity

We examined the relationship between anchorage independence and FN expression as well as their association with cellular malignancy in vivo in a group of diploid cell strains and heteroploid cell lines. The cells used in this study represent seven species and a variety of tissue types, both normal and transformed. The malignant cells include tumor lines established from human cancer patients and experimental animals and cell lines transformed in vitro, either spontaneously or with oncogenic viruses or chemicals. Each cell line was assayed simultaneously for the presence of cell surface FN, for growth in methylcellulose, and for tumor formation in nude mice.

NONTUMORIGENIC CELLS: The results for nontumorigenic cells are summarized in Table I. Normal diploid fibroblast cell strains consistently showed an extensive extracellular FN matrix and were uniformly anchorage-dependent. Similarly, all of the nontumorigenic, heteroploid, fibroblast-like cell lines were FN-positive and anchorage-dependent. In contrast, the nontumorigenic epithelial-like kidney cell lines were negative for cell-surface FN, in agreement with the earlier observation that many normal epithelial cell types stain much less strongly than most normal fibroblasts (4). These FN-negative cell lines were anchorage-dependent, like the fibroblast lines.

Human skin fibroblasts established in culture by infection with SV40, as mentioned earlier, constitute an unusual group of cells with regard to their tumor-forming ability in nude mice. These cell lines do not form progressively growing tumors but instead persist as small nodules of viable cells at the sites of injection; these may last for several weeks or months and then finally regress. Of the four independently established lines that we examined in this study (LNSV, GM 638, GM 639, and SV80) (see Table I), the first two were FN-positive and anchorage-dependent. The latter two were both anchorage-independent but differed in their FN phenotype: GM 639 had a dense FN matrix while SV80 was predominantly FN-negative, with only a few clusters of cells in the population showing FN fluorescence. An anchorage-independent mutant (NG-8) of GM 638, selected from methylcellulose after treatment with the chemical mutagen MNNG (M. Greenberg and S. Shin, unpublished material), still retained the nontumorigenic and FN-positive phenotype of the parental cell line. Thus, these three human cell lines (GM 638, SV80, and NG-8) were anchorage-independent but nontumorigenic in nude mice; of the three, two were highly FN-positive while the third was FN-negative.

TUMORIGENIC CELLS: The results obtained from tumorigenic cell lines are summarized in Table II. Cell lines established from malignant tissue generally showed a consistent association among the three properties, in that they were all anchorage-independent, FN-negative, and tumorigenic. Of the four cell lines established from normal tissue, two were FN-negative and two were positive for an extracellular matrix; all four lines,

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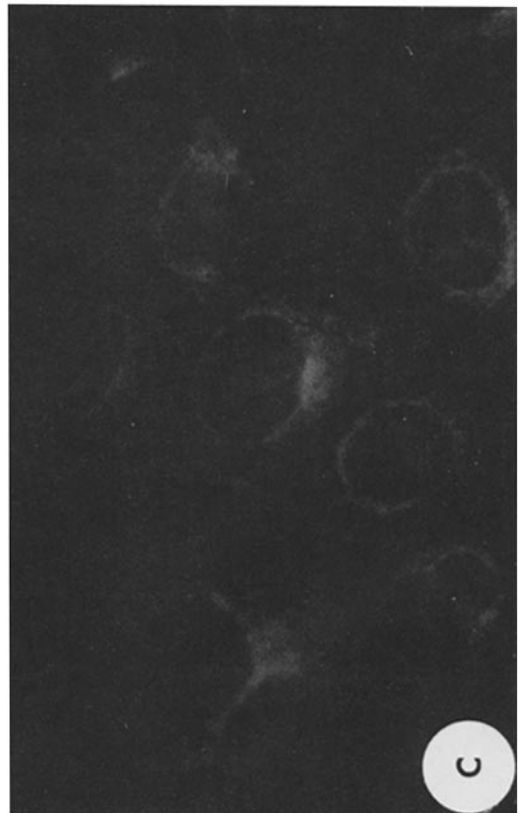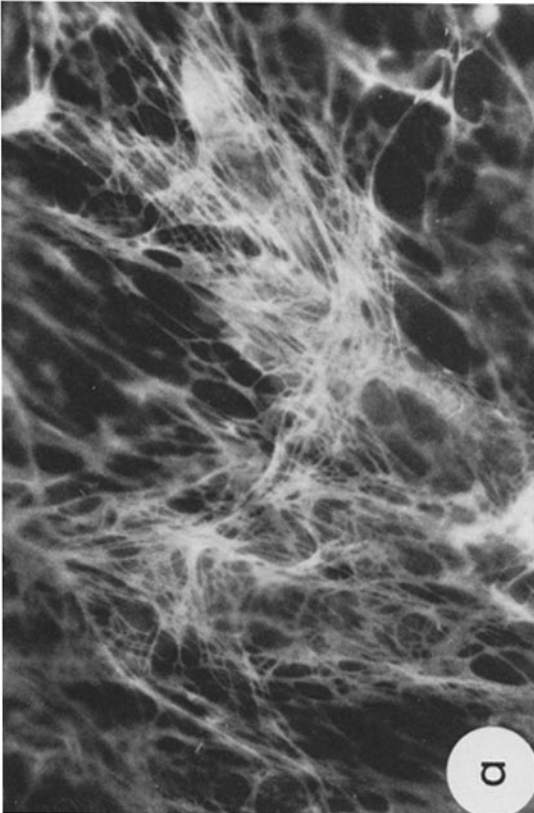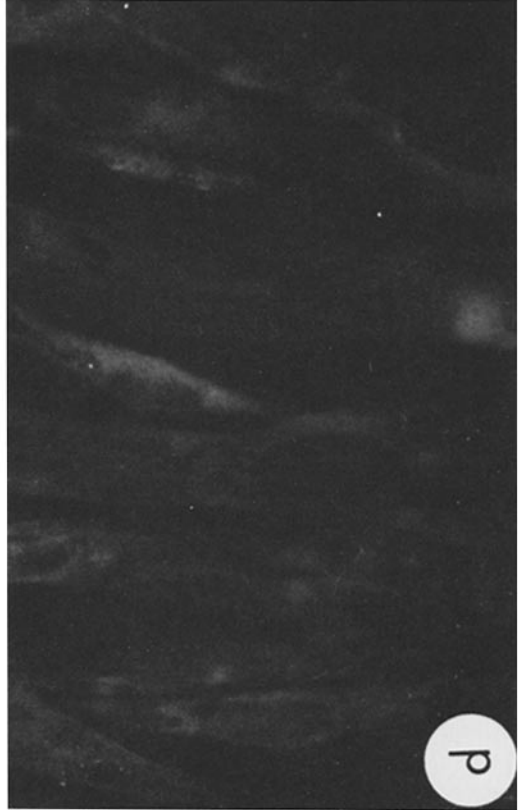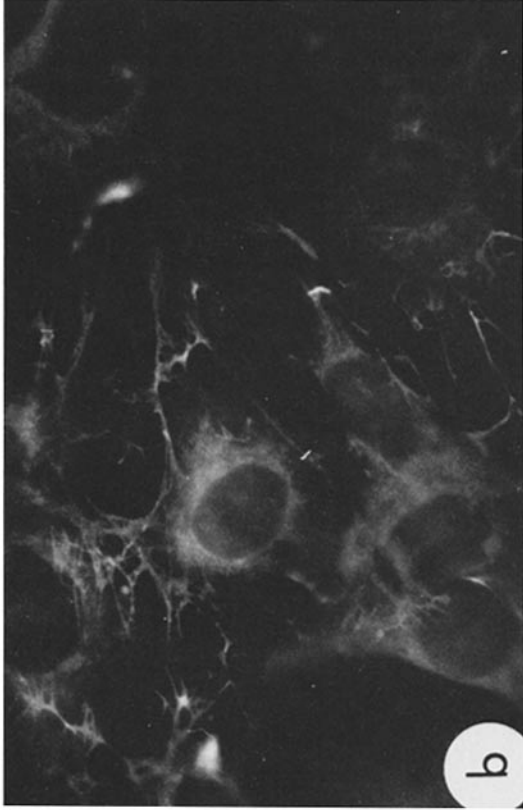
however, were anchorage-independent. Thus, the FN-negative phenotype segregated from both anchorage independence and tumorigenicity. This dissociation was also seen for the two SV40-transformed cell lines (14B and SV101) and the one polyoma-transformed cell line (PySp1A).

TABLE I

Nontumorigenic Cells

| Cells | Description | EOP in methocel | FN pattern | | Tumori-genicity |
			Extracel-lular ma-trix	Intercel-lular	
		%			
	1. Normal diploid cell strains				
CEF	Chick embryo fibroblasts	$<10^{-5}$	+		0/3
MT	Human neonatal skin fibroblasts	$<10^{-5}$	+		0/6
HEF	Human embryonic fibroblasts	$<10^{-5}$	+		0/3
MEF	Mouse embryo fibroblasts	$<10^{-4}$	+		0/7
NRF	Normal rat fibroblasts	0.001*	+		0/2
	2. Cell lines established from normal tissue				
MDBK	Normal bovine kidney cells	$<10^{-5}$	−	−	0/2
CV-1	Normal monkey kidney cells	0.0001*	−	−	0/4
NIL-8	Syrian hamster embryo fibroblasts	$<10^{-5}$	+		0/8‡
BALB/3T3	Mouse embryo fibroblasts	$<10^{-3}$	+		0/4‡
Rat-1	Established rat embryo fibroblasts	0.006§	+		0/4‡
	3. Human cell lines established by infection with SV40				
LNSV	SV40-transformed derivative of skin fibroblasts from patient with Lesch-Nyhan syndrome	0.025	+		0/20‖
GM 638	SV40-transformed derivative of skin fibroblasts from patient with galactosemia	0.014	+		0/4‖
NG-8	Anchorage-independent clone of GM 638 obtained after mutagenesis with MNNG	3.0	+		0/4‖
SV80	SV40-transformed derivative of skin fibroblasts from patient with Fanconi's anemia	1.6	±¶		0/20‖
GM 639	SV40-transformed derivative of skin fibroblasts from patient with galactosemia	18.2	+		0/20‖

* The colonies observed were "microcolonies," small but macroscopically visible clones which did not continue to enlarge in size.

‡ Nontumorigenic when assayed by the standard procedure (2×10^6 cells injected/subcutaneous site), but sometimes gave rise to tumors when $\geq 10^7$ cells were injected per subcutaneous site.

§ Data from B. Steinberg et al. (48).

‖ Nontumorigenic even at doses of 10^7 cells/site.

¶ The population is heterogeneous for FN: most cells are completely negative but a few subclones produce a dense extracellular matrix.

FIGURE 1 Immunofluorescence photomicrographs showing the different patterns of cell-associated FN seen in this study. (a) Confluent monolayer of chick embryo fibroblasts stained with anti-CSP. This pattern is typical of the dense "extracellular FN matrix" exhibited by many fibroblast cell strains and cell lines. (b) Subconfluent culture of FL 3-8 cells, stained with anti-CSP and showing an "intercellular FN" pattern. (c) Confluent HeLa cells stained with anti-CIG, showing the FN-negative phenotype. (d) Confluent monolayer of chick embryo fibroblasts stained with pre-immune serum, showing only very low background staining over the cytoplasm.

Table II

Tumorigenic Cells

Cells	Description	EOP in methocel	FN pattern Extracellular matrix	FN pattern Intercellular	Tumorigenicity
		%			
	1. Cell lines established from tumors				
TE85A	Human osteosarcoma	ND	−		3/3
PG-19	Mouse melanoma	17.5	−	−	10/10
HeLa	Human cervical carcinoma	88.8	−	−	10/10
D98/AH2	8-azaguanine-resistant mutant derived from HeLa	20.9	−	−	4/4
GH3	Growth hormone-secreting rat pituitary tumor	ND	−	−	4/4
RAG	Mouse renal adenocarcinoma, resistant to 8-aza-guanine	37.6	−*	−	2/2
	2. Cell lines established from normal tissue				
A9	8-azaguanine-resistant mutant of L cells, a mouse connective tissue cell line	50.1	−	−	5/5
L6-D−	Clonal line of rat myoblasts	54.0‡	+		2/2
WOR-6	8-azaguanine and ouabain-resistant mutant of Chinese hamster lung fibroblasts	14.8	−	−	2/2
BHK 21	Spontaneously transformed Syrian hamster baby kidney	2.1	+		3/3
	3. Cells transformed in vitro with oncogenic viruses				
14B	Established rat line (rat-1) transformed with SV40 DNA	5.0§	+		3/3
SV101	SV40-transformed mouse 3T3	27.0	+		5/6
PySp1A	BHK 21 transformed with polyoma	10.4	+		5/5

* Negative for FN when assayed by standard procedure (coverslips fixed 2 d after cells become confluent) but highly positive for an extracellular matrix when cultures were kept at confluence for 5 d or more before fixation.
‡ Data from B. Nadal-Ginard and S. Sirota, unpublished material.
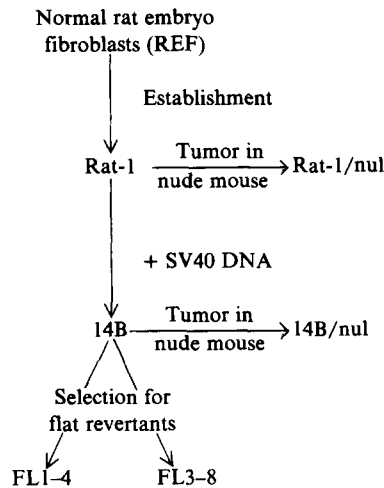§ Data from B. Steinberg et al. (48).

Dissociation of the FN Marker from Anchorage Independence and Tumorigenicity

It is apparent from the above results that in some cell lines the anchorage-independent and FN-negative phenotypes dissociate from one another and that in most of these lines it is the loss of the anchorage requirement, rather than the FN-negative phenotype, which co-segregates with tumorigenicity. To examine this finding more closely, we analyzed these three properties in a group of closely related subclones derived from rat embryo fibroblasts by specific experimental manipulations designed to alter their malignancy potentials (see Table III). Normal rat embryo fibroblasts were established in culture, resulting in the nontumorigenic, heteroploid rat-1 line (29).

After infection with SV40 DNA, clone 14B was isolated as a dense focus and was found to contain a single integrated copy of the SV40 genome (2). Revertants of 14B were isolated by selecting clones with a flat morphology (48). These cell lines were tested for growth in methylcellulose, for the presence of cell-surface FN, and for the ability to form tumors in nude mice. As shown in Table III, tumorigenicity and anchorage independence consistently co-segregated in this series of cells while the FN marker clearly dissociated. Clone 14B, the fully transformed derivative of rat-1, had a dense extracellular FN matrix. In addition, cell lines derived from the tumors of both rat-1 (which occasionally generated tumors when injected at high inoculum; see footnote to Table III) and 14B still showed a dense extracellular FN matrix, as shown in Fig. 2. Thus, cells highly positive for an

Table III

Segregation of Phenotypes in Rat Transformants and Their Revertants

Normal rat embryo
fibroblasts (REF)

|
Establishment
↓

Rat-1 $\xrightarrow{\text{Tumor in}}$ Rat-1/nul
$\qquad$ nude mouse

|
+ SV40 DNA
↓

14B $\xrightarrow{\text{Tumor in}}$ 14B/nul
$\qquad$ nude mouse

Selection for
flat revertants

FL1–4 $\qquad$ FL3–8

Cells	SV40 DNA	T-ag	EOP in methocel	FN matrix	Tumorigenicity*
			%		
REF	–	–	<0.001	+	0/3
Rat-1	–	–	0.006‡	+	0/4§
Rat-1/nul	–	–	ND	+	3/3
14B	+	+	5.0‡	+	3/3
14B/nul	+	+	ND	+	3/3
FL1–4	+	–	0.003‡	+	0/4
FL3–8	–	–	0.004‡	+	0/4

* Data from Steinberg, Boone, Shin, and Pollack (manuscript in preparation).

‡ Data from Steinberg et al. (48).

§ Nontumorigenic when assayed by the standard procedure (2 × 10^6 cells injected/subcutaneous site) but sometimes gave rise to tumors when ≥10^7 cells were injected per subcutaneous site.

FN matrix were not only tumorigenic but also retained the FN-positive phenotype even after growth in vivo as tumors.

Growth of Tumorigenic FN-Positive Cells in the Nude Mouse

The presence of a dense FN matrix in the cell lines derived from the tumors of rat-1 and 14B indicated that the passage of these two cell lines through the nude mouse was not accompanied by the selection of FN-negative subpopulations. This conclusion was further tested in a series of clonally related cell lines derived from baby hamster kidney, as shown in Table IV. The established cell line BHK 21 (27) was transformed by infection with polyoma virus (clone PySp1A), by treatment with dimethylnitrosamine (DMN) (clone DMN6A), or cloned on plastic without any treatment (cl.1 and cl.2) (N. Bouck and G. Di Mayorca, unpublished material). All of these clones were highly tumorigenic and anchorage-independent and retained essentially the same dense FN staining pattern as BHK 21. Tumors from three of these cell lines were reintroduced into culture and then retested for the expression of cell-surface FN. The strong FN staining observed with two of these tumor-derived cultures was not detectably different from the FN pattern of the original cells, while one tumor-derived line showed a marked decrease in FN staining relative to its parental cells. We therefore conclude that the growth of FN-positive cells as tumors does not necessarily involve a prior selection in vivo for FN-negative subpopulations.

Relationship Between Tumorigenicity, Anchorage independence, and Loss of FN in Somatic Cell Hybrids

To see whether segregation of the three markers in question also occurs in somatic cell hybrids, we examined selected hybrids between normal human diploid fibroblasts (which are anchorage-dependent, FN-positive, and nontumorigenic) and the highly malignant mouse cell line A9 (anchorage-independent, FN-negative, and tumorigenic), produced by H. Klinger et al. (23). These results are summarized in Table V. Many of the hybrid clones which have been tested to date are anchorage-independent and tumorigenic in nude mice. When tested by indirect immunofluorescence for the expression of cell-surface FN, some of these hybrids showed strong staining. One representative FN-positive, tumorigenic clone was passaged through the nude mouse, reintroduced into culture, and tested again for FN. As shown in Fig. 3, the tumor-derived cells showed no detectable change in the cell-surface FN pattern relative to the parental cells. These findings are consistent with the results of Der and Stanbridge (7), who recently reported that the FN-negative marker failed to segregate consistently with tumorigenicity in a series of cell hybrids between normal human diploid fibroblasts and a human carcinoma cell line.

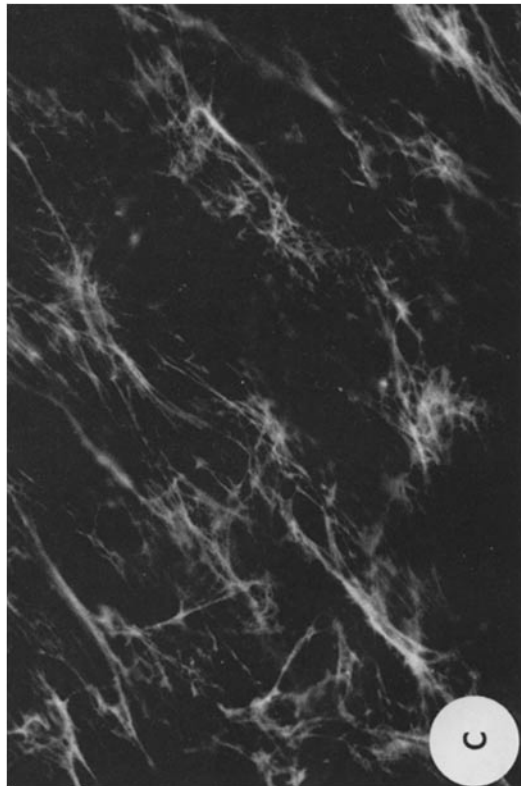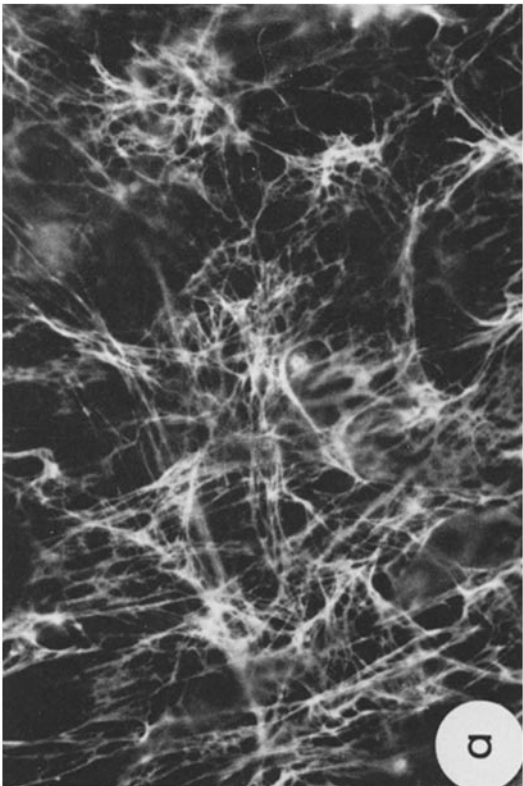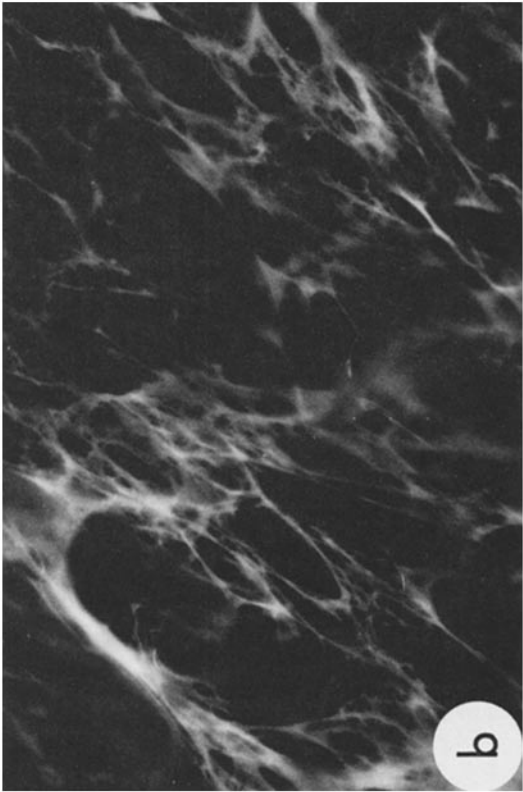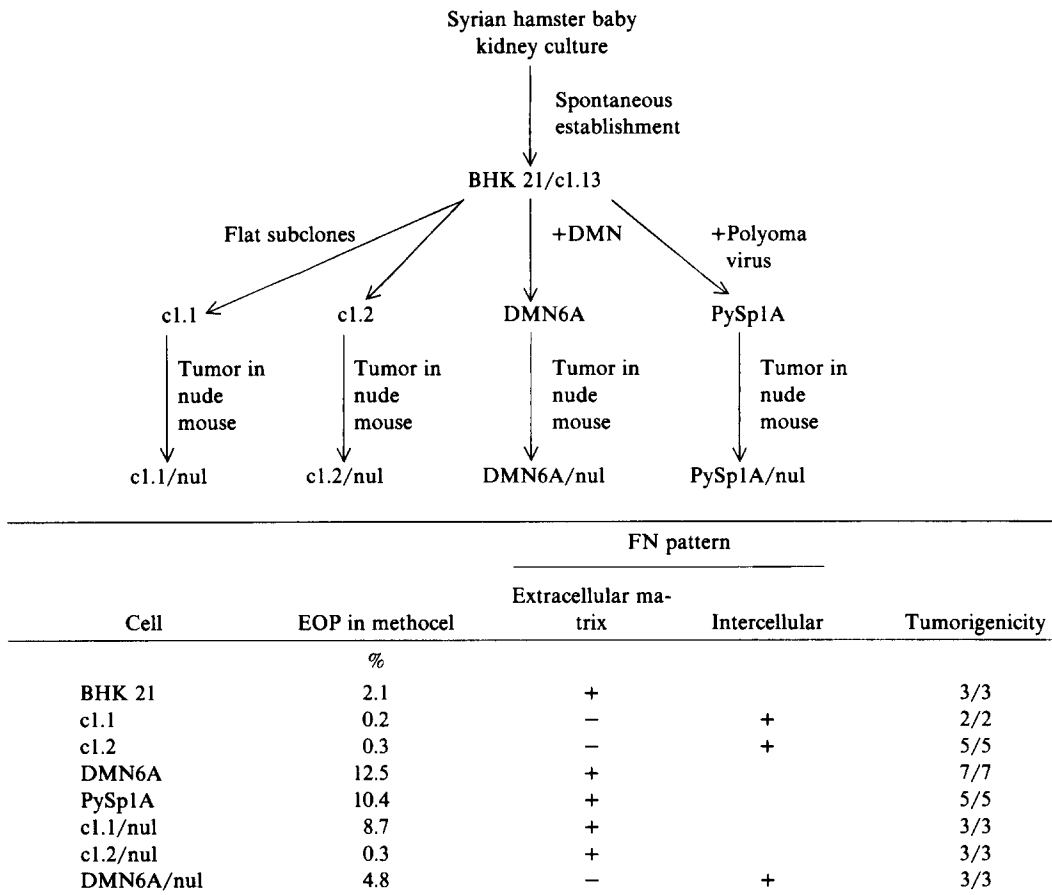## DISCUSSION

Earlier studies have shown that the loss of the

TABLE IV

Associations Between Markers in Tumor-Derived Hamster Cells

Syrian hamster baby
kidney culture

|
Spontaneous
establishment
↓

BHK 21/cl.13

Flat subclones ╱╲   +DMN ╲   +Polyoma
                                virus

cl.1 ←   cl.2   DMN6A   PySplA

Tumor in   Tumor in   Tumor in   Tumor in
nude   nude   nude   nude
mouse   mouse   mouse   mouse
↓   ↓   ↓   ↓
cl.1/nul   cl.2/nul   DMN6A/nul   PySplA/nul

		FN pattern		
Cell	EOP in methocel	Extracellular matrix	Intercellular	Tumorigenicity
	%			
BHK 21	2.1	+		3/3
cl.1	0.2	−	+	2/2
cl.2	0.3	−	+	5/5
DMN6A	12.5	+		7/7
PySplA	10.4	+		5/5
cl.1/nul	8.7	+		3/3
cl.2/nul	0.3	+		3/3
DMN6A/nul	4.8	−	+	3/3

anchorage requirement for growth in vitro is specifically correlated with cellular malignancy in vivo (11, 46). A single step selection for anchorage-independent cells from anchorage-dependent, nontumorigenic cells always led to an increased tumorigenicity; conversely, cells selected for tumorigenicity in vivo from a poorly tumorigenic cell population showed a concomitant increase in anchorage independence (46). In addition, single step selections for either tumorigenicity in vivo or for anchorage independence in vitro from a quasidiploid, nontumorigenic, anchorage-dependent

Chinese hamster fibroblast cell line (WOR-6) both selected out subclones that were near-tetraploid and shared specific marker chromosomes (24). Further evidence for a specific association between anchorage independence and tumorigenicity is provided by the work of Boone (1), who demonstrated that mouse 3T3 cells (which are normally anchorage-dependent and nontumorigenic) will form malignant tumors in syngeneic mice if the cells are first attached to glass beads. Boone's result suggests that it is only the inability of 3T3 cells to proliferate in the absence of an anchoring

FIGURE 2 Immunofluorescence patterns of confluent cultures of rat-1 and cell lines derived from it, stained with anti-CSP. (a) Rat-1; (b) 14B; (c) 14B/nul; and (d) FL 3-8. For descriptions of these cell lines, see text and Table III.
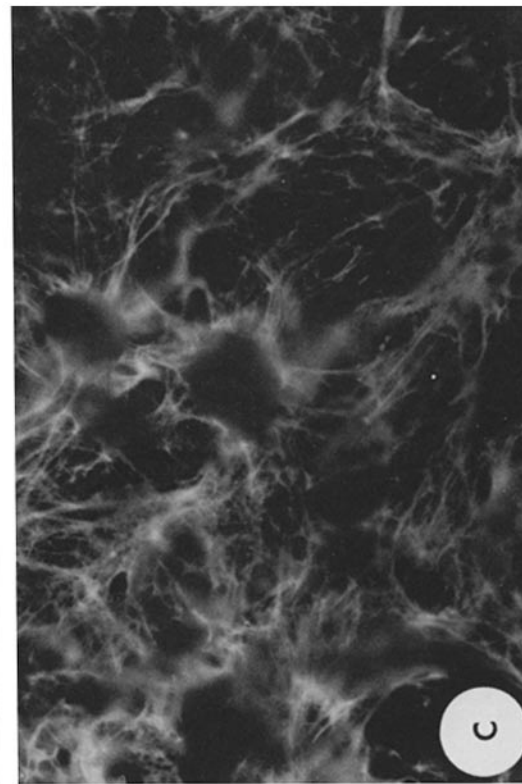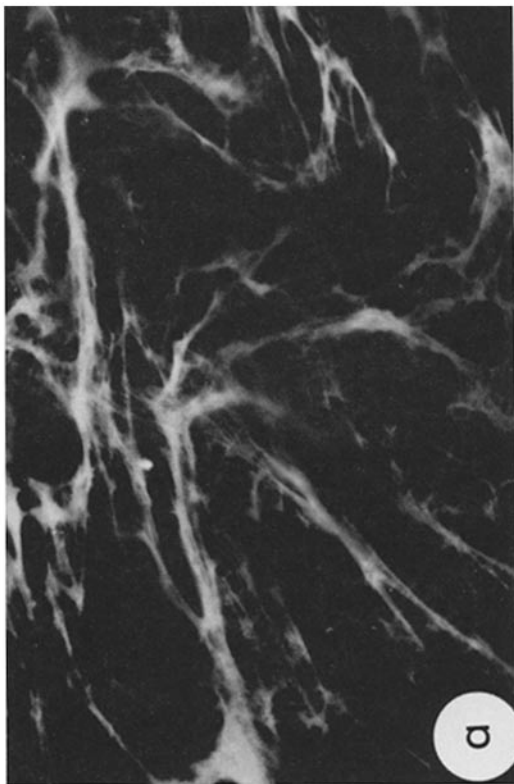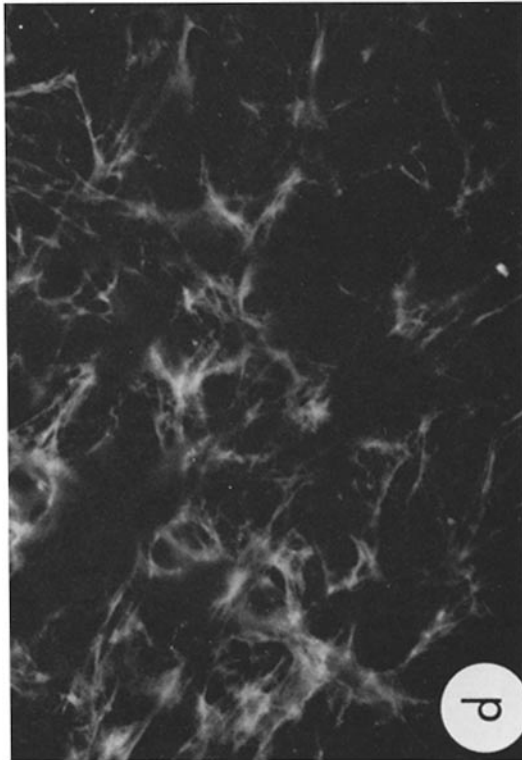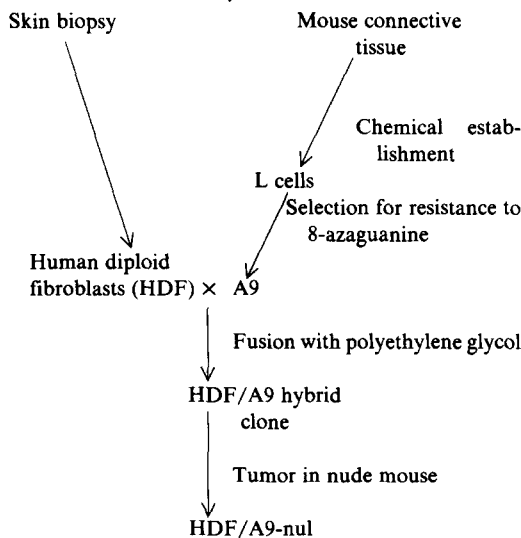
12

TABLE V

Associations Between Markers in Tumorigenic Human-Mouse Cell Hybrids

Skin biopsy   Mouse connective
                                        tissue

                                          / Chemical estab-
                                       ↓   lishment
                              L cells
                              /Selection for resistance to
                             / 8-azaguanine
Human diploid   ↓
fibroblasts (HDF) × A9

                    │
                    │ Fusion with polyethylene glycol
                    ↓
          HDF/A9 hybrid
                    │ clone
                    │
                    │ Tumor in nude mouse
                    ↓
          HDF/A9-nul

Cells	EOP in meth-ocel	FN pattern		Tumori-genicity
		Extracellu-lar matrix	Intercel-lular	
	%			
HDF	<10^{-5}	+		0/3
A9	50.1	–	–	5/5
HDF/A9 hy-brid	24.0	+		2/2
HDF/A9-nul	ND	+		3/3

substrate which prevents them from forming tumors in mice when injected as single-cell suspensions.

These experiments thus support the view that anchorage-independent growth in vitro reflects some cellular property which also determines the malignant potential of cell in vivo. Therefore, investigation of the molecular basis of the anchorage-independent phenotype offers a promising approach to the question of what specific biochemical changes in the cell lead to the acquisition of neoplastic potential.

Earlier reports from several groups seemed to indicate that in certain cell systems the loss of cel-

associated FN in vitro may be correlated with the acquisition of tumorigenicity in vivo (reviewed in reference 63). If the reduction in the cell-surface-associated FN is indeed a specific biochemical marker of malignant transformation, the hypothesis that the loss of FN is functionally related to the loss of anchorage regulation of growth would be an attractive one. It seemed to us that anchorage independence and the loss of FN could be related in one of several ways. The first possibility is that there is a direct causal relationship between these two properties—that is, the disappearance of FN from the cell surface causes a change in cellular growth control (either directly or via intermediate steps) which is manifested in vitro as the loss of the anchorage requirement and which contributes to the acquisition of neoplastic potential in vivo. However, currently available evidence argues against a direct role of FN in growth control, because neither the addition of purified FN to FN-negative transformed cells (65) nor the loss of FN from nontransformed Balb/3T3 cells (36) is accompanied by alterations in the growth properties of the cells. The second possibility is that there is no direct causal relationship between the loss of anchorage regulation and the disappearance of FN, but that these are two independent events which are both required for the cell to become malignant. If this hypothesis is correct, then each of these properties should be a necessary but not a sufficient marker for tumorigenicity in vivo. The loss of FN and anchorage independence together might then constitute the necessary and sufficient in vitro criteria of cellular malignancy. The third possibility is that the loss of the anchorage requirement and of surface FN are independent events, and that only the anchorage change is required for cellular malignancy.

These three hypotheses can be distinguished by examining which of the eight possible combinations involving the three phenotypic markers are actually observed, as summarized in Table VI. Class I contains the predominantly fibroblast-like, nontumorigenic cells which are consistently anchorage-dependent and FN-positive. Class II consists of the normal epithelioid cell lines, which are FN-negative, anchorage-dependent, and nontu-

FIGURE 3 Patterns of FN distribution in confluent cultures of cells derived from the fusion of human diploid fibroblasts and A9, stained with anti-CIG. (*a*) Human diploid fibroblasts (HDF); (*b*) A9; (*c*) HDF × A9 hybrid clone; and (*d*) HDF × A9/nul. For descriptions of these cell lines, see text and Table V.

TABLE VI

Summary of Phenotypes Observed

Class	Malignancy (tumor formation in nude mice)	Anchorage independence (colony formation in methylcellulose)	FN (presence of cell-associated FN)	No. of cell lines observed
I	−	−	+	12
II	−	−	−	2
III	+	+	−	4
IV	+	+	+	13
V	−	+	+	1
VI	−	+	−	2
VII	+	−	+	0
VIII	+	−	−	0
			Total	34

morigenic; the occurrence of cells with this phenotype argues against a direct relationship between the loss of FN and the loss of the anchorage requirement. Class III is the expected phenotype for cell lines which coordinately express the three transformed phenotypes: FN-negative, anchorage-dependent, and tumorigenic. If, however, the loss of FN is required for cellular malignancy or for anchorage-independent growth, we would not expect cells of Class IV (FN-positive, anchorage-independent, tumorigenic) to occur. In fact, we found a large number of cells in this class, including some cell lines derived from tumors in nude mice. Classes V and VI contain the nontumorigenic SV40-transformed human fibroblast lines which are FN-positive and FN-negative, respectively. Cells in which anchorage dependence and tumorigenicity are associated (Classes VII and VIII) were not found. The failure to observe cells of Class VIII (FN-negative, anchorage-dependent, tumorigenic) is inconsistent with the hypothesis that FN loss is a specific marker of malignancy or that it is directly coupled to the acquisition of anchorage independence.

It should be pointed out that the number of cell lines identified in each of the tumorigenic classes listed in Table VI does not necessarily represent their actual frequencies in all transformed cells which can grow in vitro, because several large "families" of cells which are exceptions to the correlation between FN loss and tumorigenicity have been included in this study. Yamada and Olden (63) have compiled the published data from the literature and found 77 examples in which FN was reported to be decreased or lost following

transformation, as compared to 12 cases in which it was retained. Nevertheless, our data clearly show that tumorigenic cells grown in vitro can be highly positive for cell-surface FN, even though the relative frequency of such cells compared to FN-negative tumorigenic cells may be significantly different from the sample we have studied.

Therefore, our results are consistent only with the last of the three alternatives presented earlier; that is, although the loss of FN from the surfaces of cells in vitro frequently accompanies malignant transformation, it is neither specifically associated with the acquisition of anchorage independence nor required for tumorigenicity in vivo. The presence or absence of FN on the cell surface is therefore not a reliable indicator of the neoplastic growth potential of that cell. In addition, these results confirm the conclusion reached previously that the loss of anchorage regulation of growth in vitro is a necessary but not a sufficient step in the process of malignant transformation.

Although these data appear to rule out a direct role for FN in determining cellular malignancy, the fact remains that there exists a close correlation between FN loss and malignant transformation in some cell lines (3, 16, 18). Moreover, the loss of FN has been shown to be temperature-dependent in chick embryo fibroblasts transformed with a mutant of Rous sarcoma virus (RSV) which is temperature-sensitive in its ability to transform cells in vitro (20, 40, 60). The expression of cell-surface FN in vitro is influenced by many factors, including the length of time in culture, population density, and progression through the cell cycle (3, 19, 28). Standard tissue culture systems may present the cell with an entirely different set of regulatory signals compared to those which operate in vivo. The presence or absence of FN on the surfaces of cells in vitro may not accurately reflect the role which FN expression plays in malignant growth in vivo. Examination of tumor cells in situ for the expression of FN may help to resolve this question. Alternatively, it is possible that the function which is affected by the disappearance of FN is not related to cellular malignancy per se, but instead influences the expression of some other property associated with tumor progression.

with the gel electrophoresis procedure and the immunization of the rabbits. Doctors Erkki Ruoslahti and Eva Engvall generously donated the anti-rodent fibronectin antiserum; Dr. Lan Bo Chen provided the anti-human cold insoluble globulin antiserum and taught us the staining procedure. We are grateful for their generous help. The excellent technical assistance of André Brown and Helene Weiss is much appreciated.

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