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Possible Role of Fibronectin in Malignancy

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Frozen sections of tumors induced by injecting virally transformed cells into animals were stained for fibronectin by immunofluorescence. Many tumor cell lines do not express fibronectin in tumors in situ even though some of them express fibronectin in culture. Cell shape and hormones appear to influence the expression of fibronectin in culture; however, it is unclear how fibronectin expression is regulated in vivo.

Key words: fibronectin, tumor, malignancy, cell shape, hormone, embryogenesis

In 1973, Hynes first reported that cell surface fibronectin is greatly reduced in many virally transformed cells [1]. This observation was rapidly confirmed [2]. At Cold Spring Harbor, we followed up Hynes's discovery and investigated in detail the status of cell surface fibronectin on a series of human adenovirus type 2-transformed rat cells. The remarkable feature of these cell lines (developed by one of [PG]) is that even though they are all T-antigen-positive and exhibit a spectrum of transformation phenotypes, there is a gradation of tumorigenicity among these lines. While the key to the difference in tumorigenicity among these lines may reside in the realm of immunology, we were intrigued by the observation that nontumorigenic cells express significant amounts of fibronectin, whereas tumorigenic cells express less than half of the normal level. Some highly tumorigenic cells have no detectable cell surface fibronectin. In the first phase of screening numbers of transformed cell lines, the correlation between the loss of fibronectin and tumorigenicity appeared to be good, even though we already noticed that tumorigenicity in nude mice gave a poorer correlation. For example, F19 (adenovirus-transformed rat cells) express nearly normal levels of fibronectin, are nontumorigenic in normal or immunosuppressed syngeneic rat, and yet are tumorigenic in 2 of 10 nude mice tested [3].

In order to assess whether fibronectin is really involved in the oncogenic potential of transformed cells, we have continued to investigate various aspects of the possible role of fibronectin in malignancy. Here we summarize some of our findings of the last two years.

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DOES LOSS OF FIBRONECTIN STILL CORRELATE WELL WITH ONCOGENIC POTENTIAL?

As noted in the previous summary of our work, there are transformed cells that still express significant amounts of fibronectin [4]. Some of them are highly tumorigenic – for example, mouse 3T12 cells and hamster 333-8-9 cells.

Thus, at the outset, we should say it is impossible to use the expression of fibronectin by transformed cells in culture to predict its tumorigenicity. Even in adenovirus-transformed rat cells, investigation on clonal lines isolated from methylcellulose has shown that the correlation between tumorigenicity and loss of fibronectin in culture is not absolute (Table I).

However, it should be noted that when we used epidemiologic methods to make a statistical assessment of the correlation among the 250 cell lines tested, we found the relationship between the loss of fibronectin in culture and the tumorigenicity to be extremely close (correlation of 0.92). So statistically, the loss of fibronectin in culture may still be a reasonable correlate for predicting tumor induction. But the biologic significance of such a correlation is uncertain at the moment.

FIBRONECTIN IN TUMORS IN SITU

In order to study the relationship between fibronectin and tumors properly we have to be able to study the expression of this protein in tumors in situ. Localization of fibronectin in tissue sections has been reported previously [5–7]. We satisfactorily ran through all the necessary controls and convinced ourselves that fibronectin in tissues can be unambiguously localized. The controls resulting in negative staining include: 1) FITC-conjugated IgG of rabbit anti-goat IgG alone; 2) preabsorption of goat antifibronectin antibody with purified human plasma fibronectin, prior to staining; 3) normal goat serum as a substitute for fibronectin antiserum; 4) incubation, prior to staining with FITC-conjugated second antibody, or the specimen with an excess of unlabeled IgG of rabbit anti-goat IgG, which will saturate all the antigenic sites of the first antibody (goat antifibronectin) available for RITC-conjugated second antibody (rabbit anti-goat IgG).

The key to successful immunofluorescence staining of fibronectin in tissue sections is to avoid using a second antibody that recognizes the IgG of the species of the specimen. An example of successful staining, similar to previous reports [5, 6], is shown in Figure 1, where fibronectin is localized in mouse muscle (A,B), in the stromal region but not the epithelium of the rat lung (C,D), and in blood vessels (distorted by sectioning procedures) of hamster liver (E,F). However, we are still unable to do immunofluorescence localization of fibronectin in frozen sections of skin and cornea, apparently because of nonspecific trapping.

We then procedded to stain frozen sections of tumors. All tumors tested are experimentally induced by injecting tumorigenic cells. We have not tested spontaneous or carcinogen-induced tumors. Figure 2 shows an example of immunofluorescence stain with fibronectin antibody on a frozen section of tumor induced in Swiss mice by injecting 3T12 cells. This cell line expresses a significant level of matrices in culture (C,D). However, no fibronectin can be detected in the tumor section (A,B). When the tumor was dissociated and placed in culture, the expression of fibronectin resumed and was similar to cells prior to the injection of cells into syngeneic rats. This observation indicates that fibronectin-negative cells in tumors do not result from the preferential overgrowth of fibronectin-negative cells in animals.

TABLE I. Fibronectin Expression and Tumorigenicity

Parent cell lines and methylcellulose subclones	% Fibronectin-positive cells at time of inoculation	Syngeneic as rats[a]		2- 3-week old nude mice; Route of inoculation:	
		Newborn	ATS-immunosuppressed[b]	IP	SC
F4 Parent	63	0/20	5/6	6/6	5/5
F4 M1	5	0/5	5/5	3/3	3/3
F4 M2	70	0/5	0/5	2/3	0/6
F4 M6	3	0/5	4/5	3/3	3/3
F4 M7	8	0/5	5/5	3/3	3/3
F4 M9	0	0/5	5/5	3/3	3/3
F4 M10	27	0/5	5/5	3/3	3/3
F4 M12	4	0/5	4/5	3/3	3/3
F4 M13	0	0/5	5/5	3/3	3/3
F17 Parent	100	0/20	0/10	0/6	0/10
F17 M1	32	0/5	0/5	3/3	2/3
F17 M2	78	0/5	0/5	3/3	4/5
F17 M3	85	0/5	0/5	2/3	1/3
F17 M6	17	0/5	0/5	3/3	2/3
F17 M7	7	0/5	0/5	2/3	1/3
F17 M8	0	0/5	0/5	3/3	4/4
F17 M9	0	0/5	0/5	4/4	3/4
F19 Parent	100	0/20	0/10	3/3	5/5
F19 M1	0	0/5	0/5	2/3	2/5
F19 M2	5	0/5	0/5	2/6	1/6
F19 M3	0	0/5	4/5	6/6	3/3
F19 M4	0	0/5	0/5	0/6	0/6
F19 M5	90	0/5	0/5	2/3	0/3

[a]All animals received 2×10^6 cells in 0.1 ml Ham's F10 medium.
[b]ATS given IP when rats were 8, 9, 10, 11, and 12 days old.
Adapted from Gallimore et al [17, 18].

Table II summarizes the results of immunofluorescence stain of frozen sections of various tumor. Most tumor cells do not express fibronectin in situ, even if they express fibronectin in culture. It is important to note that all these tumors are small (less than 5 mm in diameter) and poorly vascularized. In some sections blood vessels are found and, of course, are fibronectin-positive, but adjacent tumor cells are still negative. In a large tumor (2 cm in diameter) normal stromal fibroblasts frequently participate in tumor formation. For example, tumors induced in nude mice by HeLa cells often consist of as much as 70% host normal cells (Stiles, C., personal communication). Often in these tumors the fibronectin-negative tumor cells and fibronectin-positive stromal fibroblasts are intermingled. All tumor sections studied here used tumors of small size, thus minimizing the incidence of normal cell participation.

It is most noteworthy that 3T6 and 3T12 cells express significant levels of cell surface fibronectin in confluent culture yet produce no detectable fibronectin in frozen sections of their tumors. It is also important to note that some of the tumor cells in tumors induced by 333-8-9, Herpes simplex virus (type II)-transformed hamster cells, do express some detectable fibronectin (Fig. 3). So far this is the only definitive exception in which fibronectin can be localized unambiguously in tumor cells in situ. Work in progress indicates

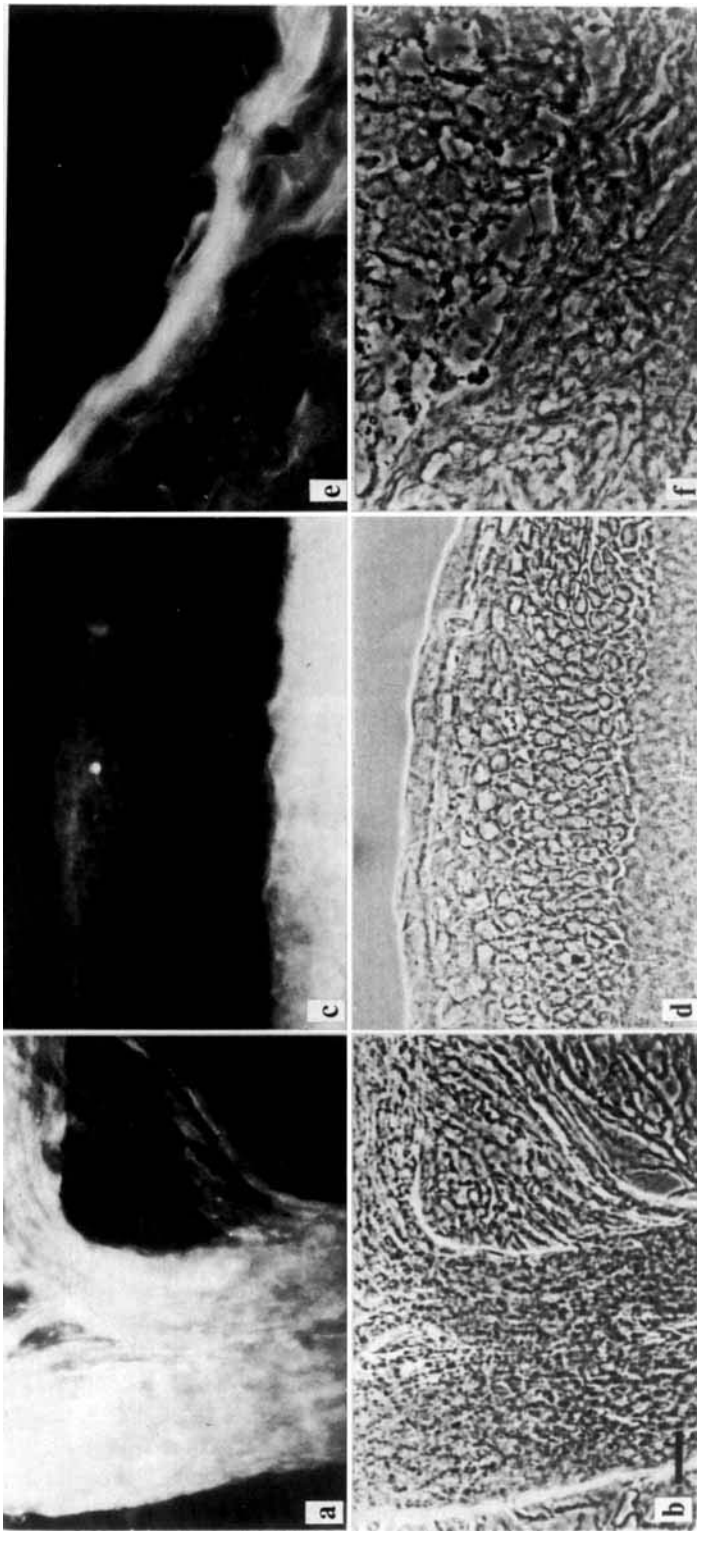

Fig. 1. Immunofluorescence stain of fibronectin in frozen section of normal tissue. (a,b): Mouse muscle; (c,d): rat lung; (e,f): hamster liver. Bar represents 15 μm.

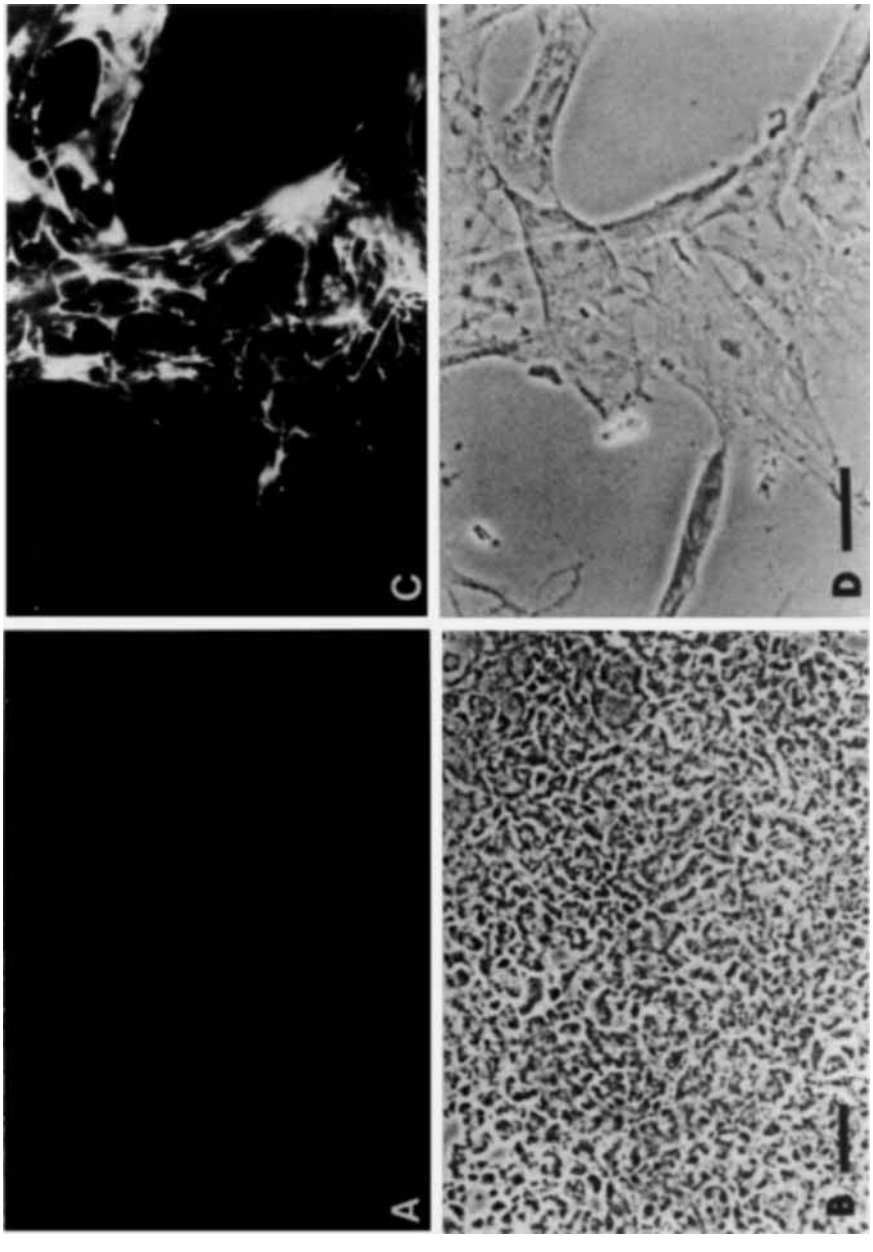

Fig. 2. Immunofluorescence stain of fibronectin in mouse tumor induced by 3T12 cells (A,B) and in culture 3T12 (C,D). Bar in B represents 15 μm and in D represents 10 μm.

TABLE II. Fibronectin in Tumor Frozen Section

Cell lines	Solid tumor induction	Metastasis to:	Fibronectin matrices in culture	Fibronectin in tumor
333-8-9, hamster	+	None	+	+
SV-3T3, mouse	+	None	+	–
Py-3T3, mouse	+	None	+	–
3T6, mouse	+	None	+	–
3T12, mouse	+	None	+	–
SVRE, rat	+	None	+	–
THE, hamster	+	Intestine, liver, kidney	–	–
2Tul, hamster	+	Lung, intestine	–	–
CCL49, hamster	+	Lung	–	–
MSV-3T3, mouse	+	Intestine, lung, liver	–	–
CCL47, rat	+	liver, stomach, intestine	–	–
T2C4, rat	+	Liver, lung, intestine	–	–
T8, rat	+	Liver	–	–
F4M1, rat	+	Liver	–	–
F4M9, rat	+	Liver	–	–
F4M12, rat	+	Lung, liver, lymphatics	–	–
F17M2, rat	+	Liver	–	–
F17M3, rat	+	Lung	–	–
F17M9, rat	+	Lung	–	–

that some mouse bladder epithelial cells transformed by chemical carcinogens do express small amounts of fibronectin in their tumors.

WHY IS THERE A DIFFERENCE BETWEEN FIBRONECTIN EXPRESSION BY TUMOR CELLS IN SITU AND IN CULTURE?

At this writing we offer no explanation as to why some cells will express fibrillar fibronectin in culture and, on the other hand, no fibronectin at all in tumors. For those who are trying to use cells in culture as a model for the behavior of cells in vivo, it is alarming that these two environments exert quite different effects on the cells. To extrapolate observations made on cells in culture to cells in vivo may be more difficult than anticipated. Fibronectin may be a significant example.

Although at the moment it is impossible to answer why there is such a difference in fibronectin expression in vivo and in culture, we offer two possible explanations to account for it. One is the influence of cell shape to fibronectin assembly; the other is the effect of hormones on fibronectin expression. It is possible that tumor cells in vivo may assume quite different shapes as opposed to fully spread cells on a plastic substratum. It is also possible that the set of hormal factors interacting with cells differs in vivo from in culture. Cells in vivo normally do not interact with serum unless there is a wound and blood clotting. Cells in culture, on the other hand, are exposed to many hormones at the same time, some of which may never be encountered in vivo with the type of cells we grow in culture. Conceivably, a different differentiation program may be induced when cells suddenly see nonphysiologic combinations of hormones in culture. It may not be surprising that in physiologic conditions tumor cells do not express fibronectin but upon the stimulation by unusual hormones in an alien environment they start to do so.

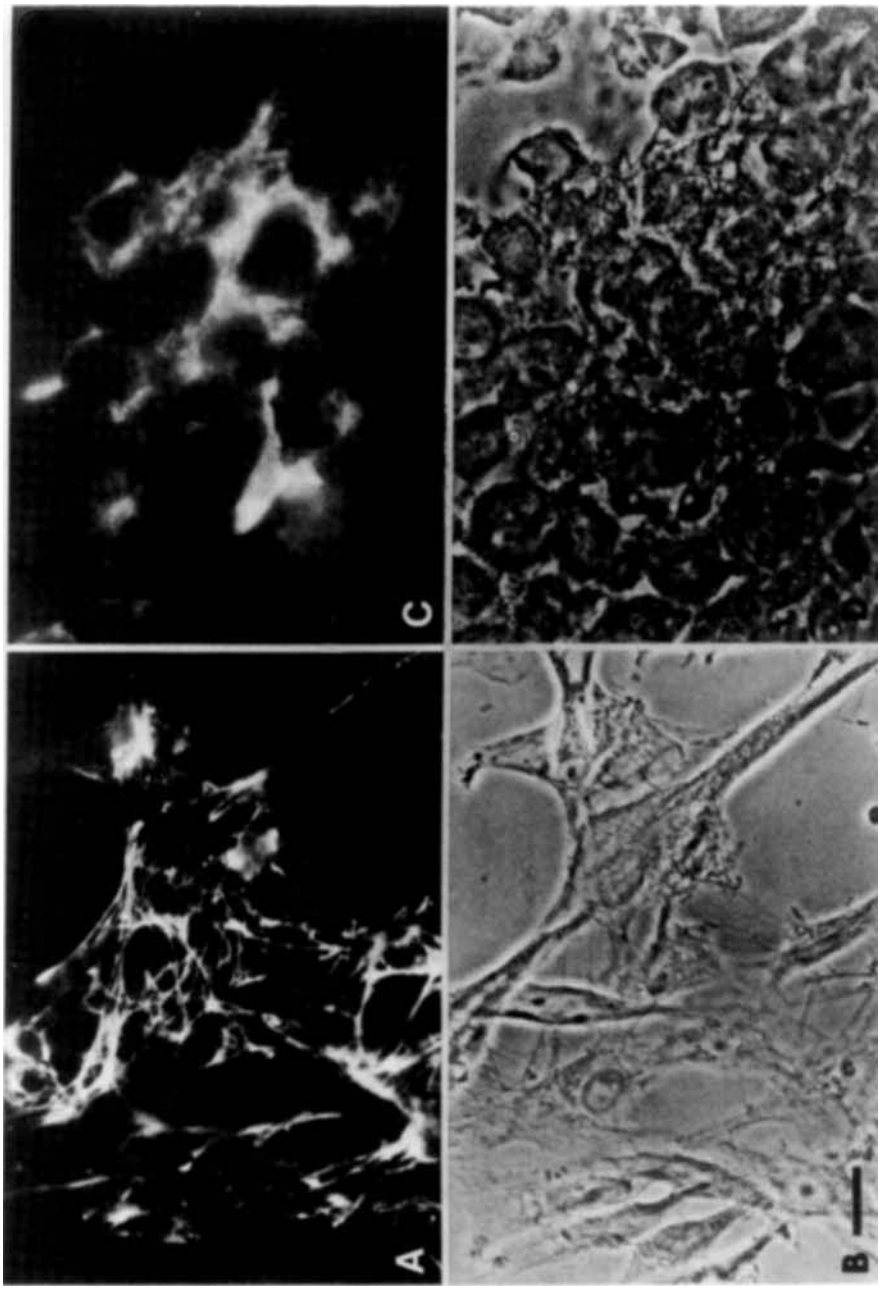

Fig. 3. Immunofluorescence stain of fibronectin in hamster tumors induced by injecting 333-8-9 cells (C,D) and in culture 333-8-9 cells (A,B). Bar represents 10 μm in B and 15 μm in D.

EFFECT OF CELL SHAPE ON FIBRONECTIN ASSEMBLY

Inspired by Folkman's studies on the effect of cell shape on DNA synthesis of normal fibroblastic cells and the possibility that tumor cells may assume quite a different shape in vivo than in culture, we undertook an investigation on the relationship between cell shape and fibronectin expression. Figure 4 shows that when 3T6 cells were kept round-shaped by placement on a bacterial dish, essentially no fibrillar fibronectin was to be detected by immunofluorescence with fibronectin antibody. However, when 3T6 cells were maintained in a well-spread shape by placement on a charged surface such as glass, massive networks of fibrillar fibronectin were detected by immunofluorescence. Cells (3T6) placed on a culture dish coated with a high concentration of hydron (1–10) dilution of stock poly(2-hydroxyethyl methacrylate, which is 12 g/100 ml in ethanol), which prevents cell spreading [8], also do not assemble fibronectin on the cell surface. But it returns to normal expression when 3T6 cells are grown on a culture dish coated with a low concentration of hydron, $1-10^{-4}$ dilution of stock solution, which supports fully spread cells.

INFLUENCE OF HORMONAL GROWTH FACTORS ON CELL SURFACE FIBRONECTIN

In 1977, we reported that Cohen's epidermal growth factor (EGF) stimulates the expression of fibronectin matrices in 3T3 cells [9]. Conceivably, if cells in culture interact with EGF-like hormones (EGF indeed is present in the serum) but not in vivo, then the difference in fibronectin expression in vivo and in culture can be conveniently explained. Unfortunately, little progress has been made as to what kind of hormones cells actually interact in vivo, in particular, the fibroblasts or epithelial cells that we study most frequently in culture. Thus, investigations on the effect of hormones on fibronectin expression provide only a few clues as to what extent the expression of this differentiated product of fibroblasts can be influenced by hormones.

Continuing this aspect of the work, we have now found, in collaboration with Drs. C. Scher and C. Stiles (Sidney Farber Cancer Institute), that platelet-derived growth factor (PDGF) has an effect opposite to that of EGF in that cell surface fibronectin completely disappears from Balb 3T3 (A31) cells treated with PDGF (5 ng/ml) for 24 h. It appears that both anabolic and catabolic hormones exist for the expression of cell surface fibronectin in 3T3 cells.

WHY DO IN CULTURE FIBRONECTIN-ABUNDANT TUMOR CELLS STILL HAVE ABNORMAL MORPHOLOGY IN CULTURE?

There are fibronectin-abundant tumor cells (1 week old in culture) that still have abnormal morphology and intercellular organization. These cells often overlap and criss-cross one another on the culture dish. Based on published information about fibronectin, one would predict that if fibronectin matrices were abundantly produced by the cells, the overall morphology of confluent monolayers would behave like primary cultured fibroblasts, aligned in parallel array. Why, then, do the in culture fibronectin-abundant tumor cells still have abnormal morphology and intercellular organization?

One possibility is that fibronectin matrices are not the overriding factor in cellular morphology and intercellular organization. Although they are producing fibronectin matrices, cells still have an option to assume abnormal morphology in culture. If this is the

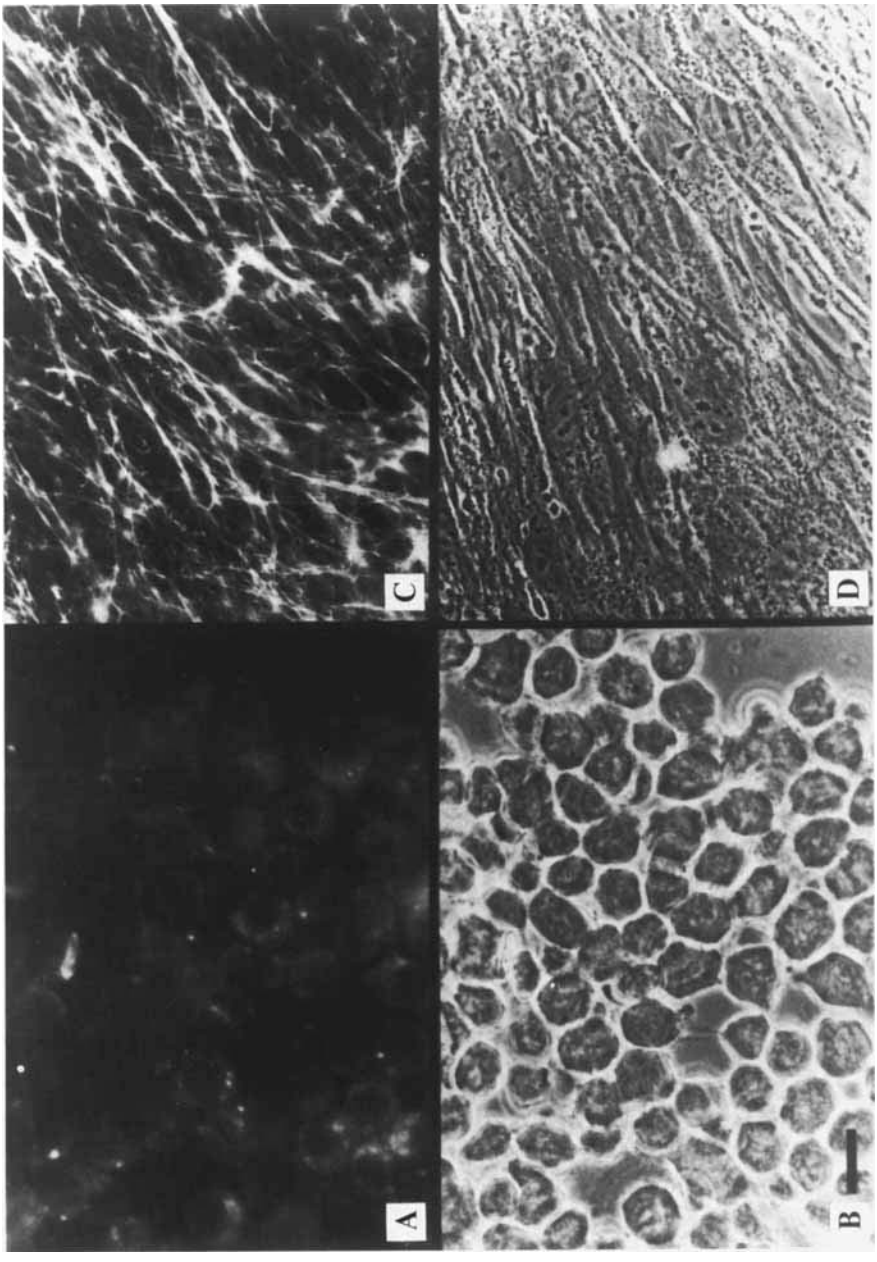

Fig. 4. Immunofluorescence stain of fibronectin in 3T6 cells one day after seeding on bacterial dish (A,B) or glass (C,D). Bar represents 15 μm.

case, one has to search for factors essential for determining cellular morphology and social behavior of cells other than fibronectin. In this regard, it is of interest to note that some mitogenic hormones such as fibroblast growth factor do alter the morphology and social behavior of 3T3 cells in a direction similar to SV-3T3 or Py-3T3 [10]. More intriguingly, conditioned medium of some of the transformed cells, which contains proteins such as sarcoma growth factor (SGF), can convert cellular morphology and intercellular organization of normal cells into transformed-like cells [11]. We have recently used conditioned medium of feline sarcoma virus-transformed mink cells (64F3clone7) to alter the morphology of chick embryo fibroblasts. When these cells were assayed for fibronectin matrices, only some alterations in distribution and organization of fibronectin matrices, but not drastic reduction in matrices, were detected (Fig. 5). Is it possible that in conditioned medium there are SGF-like molecules that influence the key "morphology regulator" which is not the fibronectin matrices? Since the effect exerted by conditioned medium takes 16 h to detect and is dependent upon protein synthesis, it is unlikely that SGF-like molecules directly interact with "morphology regulator." Rather, through hormone-like action, a new morphologic program is induced, for example, by changing gene expression. At the moment, of course, we know nothing about the molecular basis of cellular morphology. Perhaps fibronectin research represents the beginning of a long-term endeavor to understand the molecular equation of cell morphology.

While it takes 16 h for conditioned medium to induce transformed-like morphology in chick embryo fibroblasts, protease can mimic such effects within a few minutes. In most cases the loss or reduction of the fibronectin matrix is accompanied by morphologic changes [12]. However, an important exception was noted. When resting chick embryo fibroblasts were treated with highly purified thrombin they underwent a morphologic change similar to certain types of oncogenic transformation without losing or altering fibronectin matrices [13]. This is another example indicating that cells can undergo transformation-like morphologic change without affecting fibronectin matrices. Perhaps, one can conclude that the correlation between fibronectin matrices and the normal intercellular organization of cultured cells is not absolute. Therefore, one is not surprised to find fibronectin-abundant tumor cells still having abnormal morphology.

Although the above discussion tends to argue against a dominant role for fibeonectin matrices in cellular morphology, the involvement of fibronectin in morphology, intercellular organization, and "social behavior" of cultured cells is beyond any doubt [14, 15]. The question is what role it plays. Is it the cell-substratum adhesion, as suggested by numerous reports, see review by Yamada and Olden [16]?

IS THERE A ROLE FOR FIBRONECTIN IN MALIGNANCY?

In regard to human cancer, the role of fibronectin is not too encouraging. Since the normal epithelium of adult tissues rarely has fibronectin associated with it, it is difficult to argue about the significance of the "loss" of fibronectin. However, during metastasis, transformed cells have to penetrate the various basement membranes, which often contain fibronectin; this interaction of tumor cells with fibronectin may be significant in the development of a malignancy. In order to study this we have to first understand the structure and chemistry of basement membranes; unfortunately, we know very little.

For experimental tumors, where fibroblasts are often the host for oncogenic transformation, fibronectin is clearly a good cell surface marker to follow. But does it have a

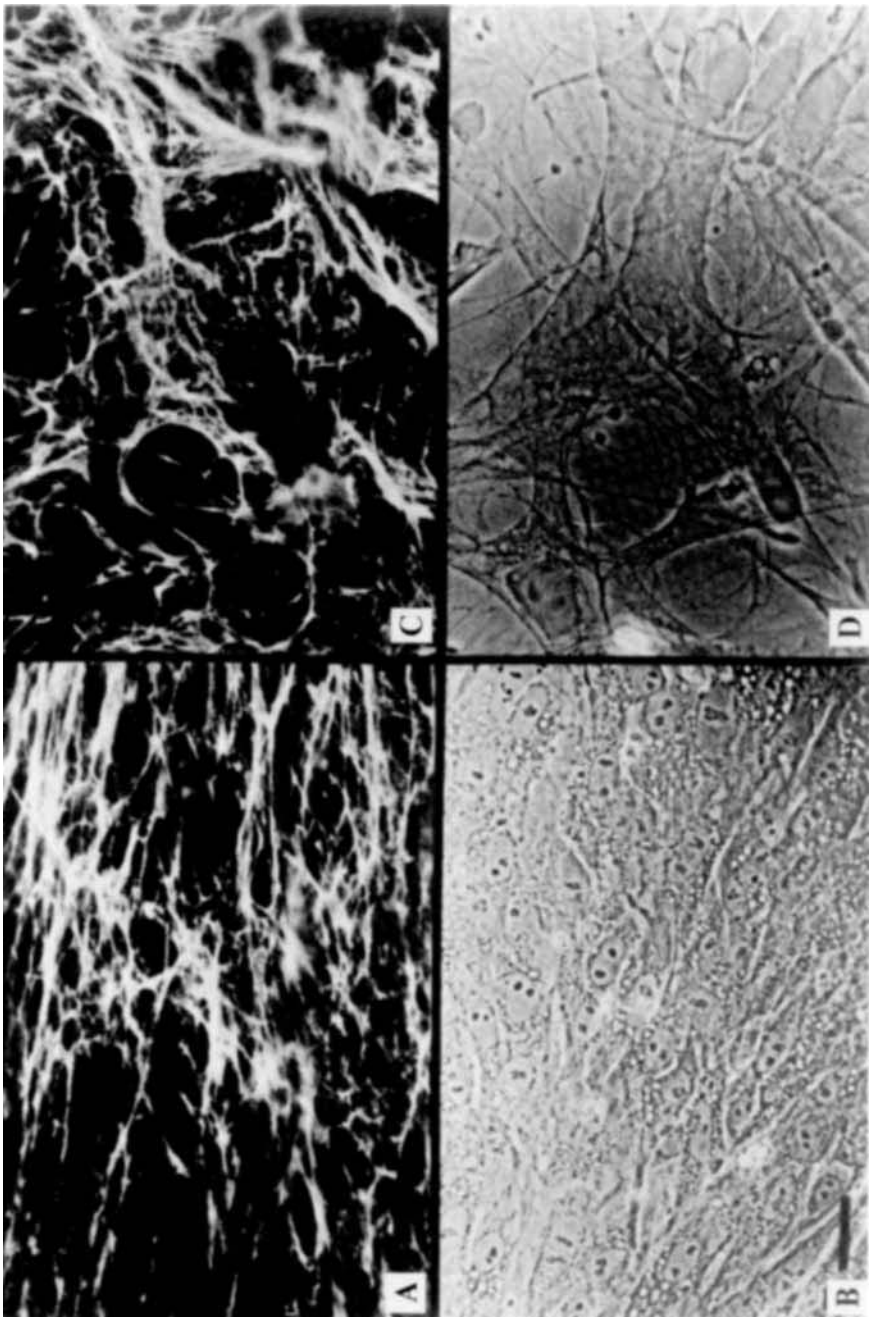

Fig. 5. Immunofluorescence stain of fibronectin in chick embryo fibroblasts (A,B) or treated with conditioned medium of FeSV-transformed mink cells (C,D). Bar represents 15 μm.

role in malignant development? The answer seems to depend on how the untransformed normal parent cells behave in vivo. At present, we don't know how cells such as 3T3, CEF, Nil or BHK, that produce fibronectin in culture behave with respect to fibronectin expression in a physiologic environment. Perhaps one can place 3T3 cells on glass beads, inject or implant them subcutaneously into animals, and then examine whether fibronectin is expressed. If that is true, then the fact that SV-3T3, Py-3T3, 3T6, and 3T12 do not express fibronectin in their tumors is of the utmost significance from an oncogenic transformation standpoint. We really need such data before we can assess whether there is any role for fibronectin in clinical tumors.

In in vitro studies of oncogenic transformation in the cell system, fibronectin is undoubtedly involved. However, as mentioned earlier, we still don't know how. But the rapid progress in this area may soon lead us to an understanding of the pathway of oncogenic transformation induced by either src gene product of T antigen. We are most hopeful that the role of fibronectin in that process will then become clear.

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