A major glycoprotein found on the cell surface of fibroblastic cells, endothelial cells, myoblasts, and some epithelial cells (see reviews in references 6, 9, and 11). This protein is sensitive to trypsin, and the amount of fibronectin on the cell surface is generally reduced upon oncogenic transformation. Antiserum raised against plasma fibronectin or extracted fibronectin has been used to localize cellsurface fibronectin by immunofluorescence, which in general reveals a three-dimensional matrix consisting of a fine fibrillar structure.

We are interested in studying the biological function of fibronectin in vitro and the interaction of tumor cells and differentiating cells with the extracellular fibronectin matrix. After accomplishing the first step of isolating a cell-free extracellular matrix containing fibronectin (2), it occurred to us that it is necessary to prepare a stable, sensitive probe of high fluorescence intensity for the studies of fibronectin in live cells. Immunofluorescence localization with fibronectin antibody has adverse effects on living cells, and some of the fibronectin matrix is inaccessible to antibody.

One of the biochemical properties of fibronectin is its high affinity for collagen. Gelatin affinity chromatography has been used to purify fibronectin from conditioned medium (3). We used this fact and conjugated gelatin with fluorescein molecules. In this report, we demonstrated that fluorescein-conjugated gelatin is a useful probe for studying cell surface fibronectin in living cultured cells.

**MATERIALS AND METHODS**

**Cell Cultures**

Chick embryo fibroblasts were prepared according to Rein and Rubin (8). Other cell lines used were: CCL47, a Rous sarcoma virus-transformed rat cell line; NIL, a hamster fibroblast cell line; HSV-NIL, hamster sarcoma virus-transformed hamster cells; Rat 1, a rat fibroblast cell line and Swiss 3T3, mouse fibroblast. All cells were grown on 12-mm glass coverslips in Dulbecco’s modified Eagle’s medium (DME) supplemented with 10% fetal calf serum.

**Fluorescein-conjugated Gelatin**

Gelatin (20 mg/ml) was conjugated to fluorescein isothiocyanate (40 μg/ml) (N. L. Cappel Laboratories Inc., Cochranville, Pa.) in 0.1 M carbonate-bicarbonate buffer (pH 9.3) at room temperature for 18 h. Unreacted dye was removed by dialysis and gel filtration through a G-25 Sephadex column in balanced phosphate-buffered saline (PBS). Fluorescein isothiocyanate-conjugated gelatin (FITC-gelatin) was stored in aliquots at −20°C.

**Live Cell Staining with FITC-Gelatin**

Cells on coverslips were washed in DME, then incubated with 20 μl of FITC-gelatin (1 mg/ml) at 37°C for 30 min. The coverslips were washed in DME and
mounted on a rubber chamber containing DME supplemented with 10% fetal calf serum. If counterstaining with antibody was required, the coverslip was further stained with 10 μl of 1:40 dilution of rabbit antifibronectin antibody and 20 μl of rhodamine-conjugated goat anti-rabbit IgG (1:10) before mounting on the rubber chamber. The specificity of the antibody to fibronectin has been described previously (1). In Fig. 4, coverslips were first stained with FITC-gelatin as described above. After the excess stain was washed off, the coverslips were fixed in −20°C acetone for 2 min, washed in PBS, and then the antifibronectin antibody was applied.

**Binding of 125I-Gelatin on Fibronectin Matrix**

Gelatin was radiolabeled with chloramine-T according to Greenwood et al. (4). Free iodine was removed by gel filtration through a G-25 Sephadex column equilibrated with PBS. 10 μl of iodinated gelatin (1 × 10⁶ cpm) was applied to each coverslip. After incubation at 37°C for 30 min, excess 125I-gelatin was washed off and the coverslips were counted in a gamma counter.

**RESULTS**

**Matrices in Live Cultured Cells as Revealed by FITC-Gelatin at High Concentration (1 mg/mL)**

FITC-gelatin at high concentration (1 mg/ml) stains an extensive network of fibers on the cell surface of chick embryo fibroblasts (CEF) (Fig. 1). This fibrous network is continuous, dense, and consists of fibers of different apparent diameters. These fibers encircle the entire cell surface. In some cultures, many layers of fibers are detected on the cell surface. This matrix gives an image similar to that of the extracellular matrix observed by scanning electron microscopy in CEF (2).

The FITC-gelatin-stainable matrix is sensitive to trypsin (0.1 μg/ml for 10 min), chymotrypsin (1 μg/ml for 10 min), subtilisin (0.5 μg/ml for 5 min), but resistant to bacterial collagenase (100 μg/ml for 4 h), elastase (5 μg/ml for 2 h), and thrombin (10 μg/ml for 1 h). This matrix is likely to consist of a protein other than collagen or elastin. That FITC-gelatin is responsible for the staining is supported by the following evidence: (a) Pretreatment of FITC-gelatin with bacterial collagenase (5 μg/ml for 30 min) abolishes the staining potential of FITC-gelatin. (b) The FITC-gelatin that has bound to matrices can be removed by bacterial collagenase (5 μg/ml for 1 h). (c) FITC alone does not stain the matrix structure under the conditions we used for staining with FITC-gelatin. (d) None of the following FITC-conjugated proteins stain the matrix: albumin, non-immune immunoglobulin G, epidermal growth factor, fibroblast growth factor, and protein A.

Besides CEF, the FITC-gelatin-stainable dense matrix can also be detected in a variety of cultures derived from connective tissue, brain, and cardiac tissue. In established normal cell lines (3T3, Nil, BHK, Rat 1), FITC-gelatin at 1 mg/ml stains a fibrillar structure similar to the fibronectin distribution detected by indirect immunofluorescence. Most transformed cells did not have an FITC-gelatin-stainable matrix, with the exception of staining at some of the cell contact areas in adenovirus type 2 transformed rat cells.

In addition to fibroblasts, we also examined two rat myoblast cultures for an FITC-gelatin stainable matrix. In both rat L6 and B44 cells, there are FITC-gelatin stainable matrices in perfused myoblasts. There are very few FITC-gelatin-stainable fibers on myotubes. Moreover, FITC-gelatin also stains a dense matrix on the lower surface of bovine endothelial cells.

**Double Staining with FITC-Gelatin and Fibronectin Antibody in Live Cells**

Unfixed primary cultures of CEF are stained first with FITC-gelatin and then stained with rabbit antibody to plasma fibronectin and rhodamine-conjugated goat anti-rabbit IgG. In the sparse cultures of CEF, the fibronectin antibody staining is superimposed on the FITC-gelatin staining (Fig. 2). This observation confirms the prediction that fibronectin fibers would be stainable with FITC-gelatin by virtue of the high affinity between fibronectin and gelatin. In the dense cultures, however, some portions of the FITC-gelatin-stained matrices are not stainable by fibronectin antibody (Fig. 3). These fibronectin antibody inaccessible areas were observed in all living cell cultures that expressed fibronectin matrices.

It has been reported previously that much of cell surface area which is impenetrable to antibody became permeable when the cells were first fixed in acetone (7). To test whether fibronectin antibody stainable areas do indeed result from the failure of penetration by antibody but not because FITC-gelatin recognizes matrices other than fibronectin in these areas, cells are first stained with FITC-gelatin, then fixed in acetone briefly for 2 min and followed by staining with fibronectin antibody. As shown in Fig. 4, under such conditions, the two stainings are closely superimposable and the matrices that are stainable only with FITC-gelatin no longer remain. It should be noted that FITC-gelatin and fibronectin antibody apparently do not compete for the same binding site on fibronectin. Antibody to fibronectin does not block or diminish the subsequent binding of FITC-gelatin to fibronectin, and vice versa.

**Nature of the FITC-Gelatin Binding to Fibronectin**

As summarized in Table I, cells were subjected to denaturing conditions before or after binding with 125I-gelatin. The counts remaining bound were compared with those of labeled, untransformed cells. These treatments do not affect the binding of antibody to the matrix, except in the cases where fibronectin is extracted from the cell surface (i.e., β-mercaptoethanol and 8 M urea).

The gelatin-binding site on fibronectin is slightly affected by a short fixation in 3.5% formaldehyde (5 min) or in pure acetone (2 min). It can also withstand 1 h in 7% acetic acid. There is a slight decrease in the amount of gelatin that can be bound after urea treatment, which is probably caused by an extraction of fibronectin from the cell. Binding is virtually eliminated by boiling the samples for 1 min or by treatment with 5% β-mercaptoethanol for 1 h or by fixation in formaldehyde for 30 min.

After the 125I-gelatin binds to fibronectin, acetone (2 min) or 3.5% formaldehyde at the time periods used cannot remove labeled gelatin from the cell. β-Mercaptoethanol causes a loss of 50% of the counts because of dissociation of the dimer form, thereby reducing total cellular fibronectin; urea (8 M, 15 min); heat (90°C, 1 min); and acetic acid (7%, 1 h) all significantly decrease bound gelatin.

**Quantitation of the Cell Surface Fibronectin by Iodinated FITC-Gelatin**

125I-labeled FITC-gelatin is used to measure the relative amount of fibronectin on the cell surface. We find that there is a striking difference in the amount of 125I-FITC-gelatin bound on primary chick cells, untransformed established cell lines, and transformed cell lines. As shown in Fig. 5, CEF binds more 125I-FITC-gelatin than Swiss 3T3, Rat 1 cells, CCL 47 and HSV-Nil. This binding experiment suggests that on a per
FITC-gelatin-stainable extracellular matrices. Unfixed CEF cells were stained with 10 μl of FITC-gelatin for 30 min at 37°C, and the coverslips were mounted on a rubber chamber. Photographs were taken with a Zeiss photomicroscope III equipped with epi-illumination. (a-c) Three different fields in the same culture. Bar, 15 μm.
cell basis, there is more fibronectin on the cell surface of primary culture cells than on untransformed established cell lines. And there is a very small amount of fibronectin on the transformed cells tested. These data are consistent with the observation of Yamada et al. (12).

Use of a Low Concentration of FITC-Gelatin (20 μg/ml) for Localizing Fibronectin

Fig. 6 shows that it is possible to dilute FITC-gelatin to a level where only primary fibroblasts are stainable with FITC-gelatin, but not 3T3 cells. Table II shows the results of the screening of numerous cell lines and primary cell types for FITC-gelatin-stainable fibronectin and SEM-detectable extracellular matrices. The results indicate that there is an excellent correlation between the fibronectin detected by a lower concentration of FITC-gelatin and the extracellular matrices detected by SEM. Thus, it is possible to avoid the lengthy procedures of SEM by simply using FITC-gelatin. Table II also demonstrates that established cell lines, irrespective of tumorigenicity, are not stainable by a low concentration of FITC-gelatin.

Comparison of the Fate of Bound FITC-Gelatin and Fibronectin Antibody in Live Cell Culture

For a primary culture of fibroblasts, where extracellular fibronectin matrices have already accumulated, the FITC-gelatin staining of the extracellular fibronectin is very stable. Neither is the FITC-gelatin released from the matrices nor is the turnover of the fibronectin matrices occurring at a significant enough rate to lead to loss of staining. When these matrices were stained with fibronectin antibody, a similar observation was made.

Fig. 7a and b show that in confluent CEF where SEM-detectable fibronectin matrices are abundant, the staining of either FITC-gelatin or fibronectin antibody is stable in culture for 10 d. In contrast, the staining of fibrillar fibronectin in 3T3 cells with FITC-gelatin is stable in culture for 1 wk, while the staining with fibronectin antibody does not last in culture for...
FIGURE 3 Double staining of dense CEF cells with FITC-gelatin and fibronectin antibody. (a) FITC-gelatin, (b) fibronectin antibody. Both photographs were taken in the same focal plane. A lot of black shadows were seen in b. Bar, 20 μm.

longer than 24 h (Fig. 7 e). The 3T3 cells deal with the antibody as they would with unwanted cell surface complexes: some are endocytosed by cells, the others are apparently released into the medium. This observation is similar to that described for hormone-receptor complexes such as EGF and its receptor (5). It is also similar to the “capping” of fibronectin by its antibody in CEF reported by Yamada (10).

DISCUSSION
To understand the function of fibronectin, it is useful to prepare a fluorescent probe for fibronectin in live and unfixed cells. FITC-gelatin seems to be an ideal choice for fibronectin localization in living cells, for several reasons: (a) FITC-gelatin provides a significant advantage over immunofluorescence and appears, thus far, to be the best method to localize most of the cell surface fibronectin, particularly that of the cell-substratum contact area. (b) The binding of FITC-gelatin to the fibronectin matrix in living cells does not induce noticeable changes in the behavior of the cell. (c) FITC-gelatin binding with fibronectin is stable and remains associated with the CEF fibronectin matrix 10 d after staining. (d) Because gelatin can localize more fibronectin matrices than an immunological stain can, radioiodinated gelatin may be more reliable than radioiodinated antibody for monitoring the quantitative changes in extracellular fibronectin during oncogenic transformation, myogenesis, or treatments with hormones, proteases, or tumor-promoting phorbol esters.

Formaldehyde treatment of fibronectin matrices normally does not affect the binding of fibronectin antibody to the matrices. However, fixation of cells in 3.5% formaldehyde for 30 min, a commonly used procedure for immunofluorescence, invariably leads to the modification of fibronectin so that FITC-gelatin can no longer bind. Because amino groups react rapidly with formaldehyde, it is possible that one of the functional groups of fibronectin involved in binding with FITC-gelatin is an amino group. β-mercaptoethanol, known to reduce the disulfide linkage in fibronectin as well as to decrease the binding of fibronectin antibody to the matrices (7), also reduces
the binding of FITC-gelatin to fibronectin matrices. Thus, the
dimeric conformation of fibronectin maintained by disulfide
linkage seems to be essential for the binding of FITC-gelatin
onto the fibronectin fibers. The boiling of cells grown on
coverslips does not lead to the detachment or gross alteration
of cellular structure. However, treatment of CEF at 90°C for
1 min completely abolishes the capacity of fibronectin matrices
to bind to FITC-gelatin without causing a loss of fibronectin
from the cell surface (data not shown). It is possible that the
conformation of fibronectin necessary for gelatin binding is
readily altered by high temperature.

In our previous report (2), we demonstrated that extracellular
matrices with fibronectin as a major component can be detected
in CEF in culture by SEM. Since then, we have screened many
cell types and established cell lines for such matrices. The
results indicate that only primary or secondary cultures of
fibroblast, myoblast, and endothelial cells are able to assemble
such matrices within 1 wk in culture. “Untransformed” estab-
lished cell lines, including 3T3, Nil-8, Rat-1, and BHK-13, all
express matrixlike fibrillar fibronectin stainable with fibronect-
in antibody, but they do not have a significant amount of
SEM-detectable extracellular matrix after 1 wk in culture. With
immunofluorescence, the appearance of fibronectin on the cell
surface of CEF and of confluent 3T3 cells in culture for 1 wk
is indistinguishable; both have similar fluorescence intensity
and distribution. However, when Yamada et al. (12) measured
the total amount of fibronectin in CEF, they found it to be at
least 5- to 10-fold higher than in untransformed, established
cell lines such as those studied here. It is probable that simply
by producing more fibronectin, CEF accumulate sufficient
amounts of fibronectin to assemble into the thick matrices
detectable by SEM. Indeed, Fig. 5 shows that there is much
more cell surface fibronectin on CEF than on the untrans-
formed established cell lines and transformed cells tested.

**FIGURE 4** Double staining of dense CEF cells with FITC-gelatin and fibronectin antibody. FITC-gelatin-stained cells (a) were fixed
with acetone at -20°C for 2 min before applying fibronectin antibody. (a) FITC-gelatin, (b) fibronectin antibody. The two stains
were exactly superimposable. Bar, 15 μm.
Why is it possible to distinguish the amount of fibrillar fibronectin between primary cultured fibroblasts and established untransformed cell lines by SEM but not by immunofluorescence? First, the epifluorescence technique allows the

| TABLE I | Binding of $^{125}$I-Gelatin on CEF Cells with Various Treatments |
|-----------------|-----------------|-----------------|
|                | $^{125}$I-gelatin able to bind to treated cells* | $^{125}$I-gelatin remaining bound to cells after treatment‡ |
| Formaldehyde, 3.5%; 5 min | 95 | 96 |
| Formaldehyde, 3.5%; 30 min | 15 | 96 |
| β-mercaptoethanol, 5%; 1 h | 5 | 50 |
| Urea, 8 M; 15 min | 85 | 10 |
| Acetone, pure; 2 min | 80 | 85 |
| Acetic acid, 7%; 1 h | 82 | 20 |
| Heat at 90°C; 1 min | 2 | 20 |

* Confluent CEF cells on coverslip were treated with various methods listed in column one before $^{125}$I-gelatin was applied. The numbers indicate the percent of counts bound as compared to untreated control coverslip.

† Confluent CEF cells on coverslips were labeled with $^{125}$I-gelatin, then subjected to treatment listed on column one. The numbers indicate the percent of counts bound as compared to before treatment.
detection of a fibril structure as small as 5 nm in diameter, provided it has been sufficiently stained. However, because the resolution of fluorescence microscopy is ~20 nm, a fiber of 5 nm will appear to be as thick as a fiber of 20 nm. However,

### Table II

**Correlation between the Detection of Extracellular Matrix by SEM and by Low Concentration of FITC-Gelatin**

| Cell types                  | Matrix detected by SEM | Matrix detected by FITC-gelatin at: |
|-----------------------------|------------------------|-------------------------------------|
|                             |                        | 20 μg/ml | 1 mg/ml |
| Chick embryo fibroblast     | +                      | +        | +       |
| Human foreskin fibroblast   | +                      | +        | +       |
| 3T3                         | -                      | -        | +       |
| Nil-8                       | -                      | -        | +       |
| Rat-1                       | -                      | -        | +       |
| BHK-13                      | -                      | -        | +       |

SEM can only detect fibers larger than 100–200 nm, provided the fiber is located in a sparse region such as the extracellular space. If such a fiber sits directly on the cell membrane, it is difficult to resolve it against the background of glycocalyx components. Therefore, if established cell lines in culture can only express a fibrillar material <200 nm in diameter, it would escape detection by SEM. Second, the production of more of the fibronectin fibers increases the incidence of bundle formation. As shown in our previous report (2), the appearance of these bundles parallels stress fiber formation by microfilaments. In cultured CEF, fibronectin fibers cluster together to form a stress fiberlike structure. The larger diameter of these fibers increases its probability of detection by SEM.

At a lower concentration of FITC-gelatin, the profile of staining among various cell types tested is quite different from that observed at the high concentration. Only primary cultures of fibroblasts are stainable, and none of the established cell lines expressing fibronectin can be stained (Fig. 6). Because only those cells able to form extracellular matrices detectable by SEM are stainable with a low concentration of FITC-gelatin, FITC-gelatin at 20 μg/ml can be used to predict

**FIGURE 7** The fate of FITC-gelatin in CEF and 3T3. CEF and 3T3 cells were double stained, first with FITC-gelatin, then with antibody to fibronectin. The stained coverslips were returned to regular culture medium for 10 d (a and b) or 24 h (c–f) before they were examined. (a) CEF (3 d old) with fibronectin antibody for an additional 10 d; (b) CEF (3 d old) with FITC-gelatin for an additional 10 d; (c) CEF (16 h old) with fibronectin antibody for 24 h; (d) CEF (16 h old) with FITC-gelatin for 24 h; (e) 3T3 (3 d old) with fibronectin antibody for 24 h; (f) 3T3 (3 d old) with FITC-gelatin for 24 h. Bar, 40 μm.
whether a given cell type will express SEM-detectable matrices with a simple staining procedure of <10 min. In contrast, it takes at least 5 h to determine whether SEM-detectable matrices are produced by a given cell type.

Our results suggest that established cell lines, whether untransformed, transformed, or tumorigenic, express negligible amounts of SEM-detectable matrices of FITC-gelatin (at low concentrations) stainable fibronectin matrices. The loss of the ability to assemble such matrices may be a hallmark of the cell-acquired indefinite growth potential in culture. Because growing evidence indicates that immortal cell lines, even those previously thought to be untransformed, tend to be tumorigenic in animal, it appears that the loss of FITC-gelatin (20 μg/ml) stainable matrices or SEM-detectable extracellular matrices may correlate closely with oncogenic transformation.

It has been hypothesized that fibronectin is somehow involved in the maintenance of transformation phenotypes. Our results suggest that perhaps it is the lower rate of fibronectin accumulation when cell density increases that correlates with the occurrence of transformation or the increase in tumorigenicity. Because the majority of transformed fibroblast lines still synthesize fibronectin, even though at a lower rate, it is unreasonable to expect that all transformed cells should lose fibronectin completely from the cell surface. The results presented here reinforce the notion that fibronectin expression on the cell surface and its accumulation as extracellular matrices is significantly impaired in transformed cells. Perhaps, it would be better if the studies of the possible role of fibronectin in transformation were to place less emphasis on whether a small amount of cell surface fibronectin remains detectable by immunofluorescence in transformed cells and, rather, to focus on the rate of accumulation of fibronectin in extracellular matrices. After all, it is the matrix that is the destination of fibronectin as well as the environment in which cells live.

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