Behavior of Cells Seeded in Isolated Fibronectin Matrices

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ABSTRACT Cell-free fibronectin matrix (FN-matrix) isolated from chick embryo fibroblasts was used to study cell-matrix interaction. After 24 h, most fibroblastic cells, including those without cell surface fibronectin, adopted bipolar fusiform morphology. Cells grew in parallel arrays and aligned with each other apparently along FN-matrix. Since the orientation of fibronectin fibers was determined by chick embryo fibroblasts, our results suggested that intercellular organization of "matrix-using" cell type may be influenced by "matrix-producing" cell type. Whereas the elongation and alignment effects induced by FN-matrix have been detected in fibroblasts (both normal and transformed), myoblast, aortic endothelial cells, neural cell lines (B103 and RT4D1), and cardiac muscle cells, similar effects are not detected in bone marrow hemopoietic cells, circulating lymphocytic T and B cells, and sympathetic neurons. For epithelial cells, FN-matrix has varying effects. Elongation and alignment effects are detected only in transformed epithelial cells with a great reduction in keratin expression. The morphology of normal or transformed epithelial cells with abundant keratin appears unaffected by FN-matrix. FN-matrix reduced the growth of several transformed fibroblastic lines up to 25%, but did not restore the appearance of actin stress fibers and the normal migratory activities of Rous sarcoma virus-transformed rat cells.

Most adherent eukaryotic cells in vivo are bathed in an environment made of extracellular matrices. The importance of this environment in relation to differentiation, migration, morphogenesis, and disease has long been recognized (12). The matrices are composed of collagens, glycosaminoglycans, proteoglycans, glycoproteins, and other minor components. The exact functions of these components and the mode of interaction among them are still unclear. To help clarify these questions, particularly the poorly understood cell-matrix interaction, cell culture system has gradually become useful. Since the early report that fibroblasts in culture produce fibrillar materials (18), it has been recognized that several other cell types also express extracellular matrices in culture. For example, intercellular fibronectin matrices (FN-matrices) are expressed not only in cultured fibroblasts (27) but also in cultured myoblasts (4, 15), some epithelial cells (5), vascular endothelial cells (3, 16), and corneal endothelial cells (10, 26).

To understand the role of cell-matrix interaction, cell-free matrices might be useful since chemical characterization of the matrix as well as its biological effects can be conveniently studied. Thus far, FN-matrices from chick embryo fibroblasts (6) and human foreskin fibroblasts (13), and extracellular matrices containing collagen, fibronectin, and laminin from corneal endothelial cells (10) have been isolated, characterized, and used for studying cell-matrix interaction. In this report, we describe the effect on the elongation and alignment of transformed and normal fibroblastic cells of cell-free fibronectin matrices isolated from chick embryo fibroblasts. The effects of FN-matrix on cell growth, actin stress fibers, and migratory activities of tumorigenic fibroblastic cells were investigated. In addition to fibroblastic cells, the interaction of FN-matrices with cardiac muscle cells, aortic endothelial cells, neural cell lines (B103 and RT4D1), bone marrow hemopoietic cells, circulating lymphocytic T and B cells, sympathetic neurons, normal epithelial cells, carcinogen-transformed epithelial cells, and carcinoma-derived cells was also studied.

MATERIALS AND METHODS

Cell Cultures: All cells were cultured in Dulbecco's modified Eagle's medium (DME) supplemented with 5% calf serum (Gibco Laboratories, Gibco Div., Grand Island, NY). Primary chick embryo fibroblasts (CEF) were prepared according to Rein and Rubin (19). Other cell lines used were: CCL47, a Rous Sarcoma Virus (RSV)-transformed rat cell line, and CCL64, a mink fibroblast line (American Type Culture Collection, Rockville, MD; AnAn, established from a tumor induced by injecting RSV into newborn Lewis rat and passaged in vivo three times (23); i.e. RSV-Rat, Rat 1 transformed with a temperature-sensitive mutant in src gene of RSV-B77, established by J. Wyke (Imperial Cancer Research Fund, England) and provided by R. O. Hynes (Massachusetts Institute of Technology); SV0, SV40-transformed human fibroblast, from D.
RESULTS

Morphological Effect of the FN-Matrix

Soluble fibronectin has been shown to restore normal morphology to most but not all transformed cell lines (1, 30, 31). AnAn, a rat tumor line induced by Rous sarcoma virus and without detectable cell surface fibronectin, is unresponsive to soluble fibronectin with regard to morphological reversion. However, after 2 d in cell-free FN-matrix, AnAn cells are bipolarized to adopt fusiform morphology and aligned in parallel array (Fig. 1). Fig. 2 is a scanning electron micrograph of AnAn cells growing on glass coverslips with or without FN-matrix. As seen, FN-matrix changes the shape of AnAn cells drastically. The three elongated cells were bipolarized in the direction apparently along the orientation of fibronectin fibers. Besides AnAn cells, FN-matrix also affects the morphology and alignment of a variety of cell lines and cell types (Fig. 3). The bipolarization effect of the matrix is detectable on transformed cells even at a high density (Fig. 3f). The effects of FN-matrix survive the pretreatments of matrix with bacterial collagenase (37°C for 48 h), air drying (22°C for 5 min), or heat (80°C for 5 min). It is, however, very sensitive to trypsin treatment (0.5 μg/ml for 5 min).

Spreading and Attachment

We used time-lapse cinematography (16-mm film) to compare the spreading of CCL47 cells on plastic substratum to those on FN-matrices. Although CCL47 is RSV-transformed rat cell, within 24 h after seeding on plastic the spreading and morphology of this cell line are similar to those of normal cells. In the absence of FN-matrices, CCL47 cells attach to the dish within 15 min. By 30 min, 80% of the cells spread radially and have extensive marginal ruffles (lamellipodia). No bipolarity is detected in these spreading cells. Bipolarized cells are rarely detected until 12 h later. On FN-matrix, all of the cells attach to the substratum in <15 min. However, spreading does not occur immediately. By 2 h, only 40% of the cells spread out, but marginal ruffles are not readily detectable. Cells appear to be bipolarized soon after spreading. By 5 h, 80% of the attached cells have spread out, and most of them are also bipolarized.

A series of phase-contrast photographs on the spreading of CCL47 cells on plastic substratum and on FN-matrix is shown in Fig. 4. The results on the spreading are identical to those obtained with 16-mm time-lapse cine. 10 min after plating CCL47 cells on FN-matrix, the first photograph was taken (Fig. 4a). Identical positions in these figures are marked as reference points by arrows, open or closed squares, circles or numbers. In Fig. 4a, cell no. 1 starts to spread, but no marginal ruffle is seen; two pairs of cells marked no. 2 and 3 are still in round shape. On the plastic dish at 10 min, many cells are spreading radially and marginal ruffles are readily detectable (cells no. 4 and no. 5 in Fig. 4b). By 40 min, many cells start to spread on FN-matrices, but the membrane extension seems to be inhibited. Marginal ruffles are rarely detected. Cell no. 1 is bipolarized as it spreads out. On the plastic dish, cells no. 4, 5, and 6 remain radially spread at 40 min. 95% of the spreading cells do not have a defined bipolarity. By 2 h, more bipolarized cells are seen on FN-matrix, and cell pair no. 2 starts to spread out (Fig. 4e). On the plastic substratum (Fig. 4f), membrane extension of spreading cells almost reaches the maximum. Cells no. 5 and 6 are fully spread out at this time point, yet still without bipolarity.

By 5 h, 95% of the cells on FN-matrix are spread out, and almost all of them are bipolarized. Cells within each cell pair, no. 2 and 3, are bipolarized in a parallel direction; cell no. 1 is fully bipolarized (Fig. 4g). On the plastic dish some cells start to gain their bipolarity (Fig. 4h). One of the two cells in pair no. 5 is partially bipolarized, but not the other. Cell no. 6 is bipolarized and starts to migrate towards the white dot marked by a small open circle. Cell no. 4 still remains radially spread. By 12 h, cells on FN-matrix are extending their processes. Cell no. 1 is migrating away from the open square and in the same direction as the initial bipolarity; all cells in each pair, no. 2 and 3, are extending their processes (Fig. 4i).
FIGURE 1 Phase-contrast micrographs of AnAn (RSV-rat tumor) cells on (a) plastic dish and (b) cell-free fibronectin matrices. Bar, 120 μm.

FIGURE 2 Scanning electron micrographs of AnAn (RSV-rat tumor) cells on (a) cell-free fibronectin matrices and (b) glass coverslip. Bar, 35 μm.
cell no. 4) remaining radially spread without obvious bipolarity (Fig. 4j).

By 24 h, cells on FN-matrix remain bipolarized with long processes. Cell no. 1 has moved farther away from the open square. Both cells in pair no. 2 have divided and the resulting four daughter cells are also bipolarized with processes (Fig. 4k). On the plastic dish, cell no. 6 has migrated farther down, and only by this time are some of the cells partially polarized (Fig. 4l).

Taking these observations together, it appears, therefore, that FN-matrix may delay cell spreading, inhibit radial membrane extension, and promote bipolarization.
Migration

In view of the above results, it is of interest to compare, by time-lapse cinematography, the migrations of cells seeded on substrata with or without FN-matrices. FN-matrix did not promote migration of cells such as AnAn and CCL47, which normally have little displacement on plastic. It appears that bipolarization of these cells by FN-matrix did not improve them with respect to undertaking migration. However, cells that normally migrate on plastic dishes, such as primary fibroblasts of various origins, tend to move along the fibers of FN-matrix. The guiding effect of FN-matrix is most readily detectable in CEF. On plastic substratum, CEF migrate randomly. On FN-matrix, the migration pathway of CEF tends to follow the fibronectin fiber.

Cytoskeleton

It has been reported that soluble cellular fibronectin induces the formation of actin-containing stress fibers in transformed cells (1, 29). We therefore examined whether stress fibers can also reappear in RSV-transformed rat cells grown in FN-matrices. The staining of actin in AnAn cells growing on plastic substratum is diffuse, whereas filament bundles were detected in untransformed Rat-1 cells with actin antibody (Fig. 5 a and b). The staining of actin in bipolarized AnAn cells growing on FN-matrix is still diffuse, and no stress fiber is detected (Fig. 5 c).

Growth

Fibronectin matrix has little effect on the growth of untransformed fibroblastic cell lines (i.e., Rat 1, 3T3, and CCL64). These cells have a similar growth rate whether grown on FN-matrix or not. The growth of secondary chick embryo fibroblasts and rat embryo fibroblasts is slightly enhanced when they are seeded on FN-matrix. For transformed cells, unexpectedly, the FN-matrix has negative effects on their growth. Up to 25% less of transformed cells grown on FN-matrix is observed when compared with those grown on plastic (Fig. 6).
These transformed cell lines include CCL47, AnAn, t.s. RSV-Rat, Py3T3, and 64F3 (FeSV-mink).

**Epithelial Cells**

The above experiments were carried out on fibroblastic cells. It is of interest to determine whether epithelial cells will respond to FN-matrix similar to fibroblastic cells. Fig. 7 shows that normal mouse bladder epithelial cells are not bipolarized by FN-matrix, and no alignment effect is detected. Since these epithelial cells are known to contain abundant keratin, and since we previously observed that many transformed epithelial cell lines with a significant reduction in keratin expression adopt fibroblastic morphology in culture (21, 22), we examined the polarization and alignment effects of FN-matrix on various carcinogen-transformed epithelial cell lines and carcinoma-derived cell lines. Table I summarizes the results and Fig. 8 illustrates an example of the effect of FN-matrix on benzo(a)pyrene-transformed rabbit bladder epithelial line, RBC-1 (22). It appears that for epithelial cells there is a correlation between a decrease in keratin content and an increase in propensity to be polarized and aligned along FN-matrix.

**Other Cell Types**

We also examined the morphological effect of FN-matrix on bone marrow cells, cardiac muscle cells, aortic endothelial cells, neural cell lines, lymphocytic cells, and sympathetic neurons. The FN-matrix promotes the adhesion and polarization of some bone marrow cells to the substratum. These adherent cells are probably histiocytes and reticulum cells. Attachment and polarization of bone marrow hemopoietic cells on FN-matrix are not detected. Rat cardiac muscle cells also become
Figure 5 Immunofluorescence staining with actin antibody of (a) AnAn cells on a glass coverslip; (b) Rat 1 cells on a glass coverslip; (c) AnAn cells on a coverslip containing cell-free fibronectin matrices; and (d) phase-contrast micrograph of (c). Bars: a, c, and d, 40 μm; b, 20 μm.

more elongated and aligned on FN-matrix. Bovine aortic endothelial cells and neural cell lines (B103 and RT4D2) are polarized and aligned by the matrix as well. Sympathetic neurons with or without nerve growth factor are not affected by FN-matrix, and the neurite outgrowth does not follow the fibronectin fibers in general. The morphology of mouse lymphocytic T and B cells is not affected by the FN-matrix.

**DISCUSSION**

We reported previously the isolation of cell-free fibronectin matrix from cultured chick embryo fibroblasts (6). In this report, we investigated further the biological effects of cell-free fibronectin matrices. It appears that fibronectin matrix is able to bipolarize all fibroblastic cells into fusiform morphology and to align them in a parallel array, similar to those seen in the histological sections of connective tissues. All tested transformed fibroblasts irrespective of the expression of cell surface fibronectin also respond to fibronectin matrix by reversing their morphology to a more normal spindle shape and aligning in a parallel array.

Whether fibronectin molecule alone is responsible for the effect of FN-matrix described here or proteoglycans are also required needs further investigation. Our rabbit anti-fibronectin antibody preparation does not block the biological effects of FN-matrix (data not shown). However, Rovasio et al. (20) have shown that the antibody against 160-kdalton cell-binding fragment of fibronectin does block the biological effect of FN-matrix on neural crest cell migration. Our antibody may not recognize the cell-binding site of fibronectin. Although actin and myosin are present as contaminants in cell-free FN-matrix (6), it is unlikely that they play a role in the effects of FN-matrix described here. These contaminants are present in glob-
FIGURE 6 Effect of cell-free fibronectin matrices on the cell growth of various cell lines and primary fibroblasts. White bars, control; cross-hatched bars, matrix.

FIGURE 7 Human breast carcinoma-derived cell line, MCF-7, on (a) a plastic dish and (b) cell-free fibronectin matrices.

ular structures randomly distributed in FN-matrix. Areas free from actin and myosin are readily detectable by immunofluorescence with actin and myosin antibodies. Fig. 5c is an example of area of FN-matrix devoid of actin but still supporting the bipolarization of RSV-rat cells. Recently, we have prepared FN-matrix from cytochalasin-B-treated cells. Very large areas of FN-matrix totally free from actin and myosin are available for studies. Cells seeded in these areas behave as do those in areas contaminated with actin and myosin. Thus, actin and myosin appear not to be involved in the biological effects of cell-free FN-matrix.

We have attempted to analyze the mechanism involved in the bipolarization and alignment of cells in FN-matrix. On the basis of the results presented in this report, the simplest explanation seems to be that FN-matrix exerts a contact guidance effect on those responding cells. The contact guidance phenomenon was first characterized by Weiss (28) and subsequently confirmed in several other studies (2, 7, 8). Time-lapse cinematography and phase-contrast micrographs taken at longer time intervals (Fig. 4) of cells seeded in FN-matrix indicate that fibronectin fibers may provide the physical structure for contact guidance. If a cell type tested is normally migratory on plastic substratum, once a contact guidance effect is exerted by fibronectin fiber the cells tend to migrate along the fibronectin fibers. However, if a cell type tested is normally nonmigratory, such as CCL47, although cells are bipolarized and aligned by fibronectin fibers the migratory activity is not enhanced. It appears as if contact guidance effect has been exerted by FN-matrix on CCL47 cells, but the migratory activity of these cells has not been improved even after the cells have become spindle-shaped.

The influence of cell shape on cell proliferation has been elegantly studied by Folkman's laboratory. The inhibitory effect of FN-matrix on the growth of some transformed cell lines observed here may be related to the findings reported by Folkman and Moscona (9). It is conceivable that, by adopting a more normal shape, transformed fibroblasts may also proliferate more like normal fibroblasts to a lower final density.
The lack of influence on the morphology of T and B lymphocytes and hemopoietic cells from bone marrow by FN-matrices may be expected since the shape of these cells is spherical, and the differentiation programs probably preclude them from spreading into spindle shapes. That nerve cells such as sympathetic neurons, which are able to send out long neurites, are also unresponsive to FN-matrices is more disappointing. Most intriguing, however, is the unresponsiveness of keratin-abundant epithelial cells to FN-matrix. It appears that the expression of abundant keratin in such cell types is related to the unresponsiveness to FN-matrix for bipolarization and alignment. Epithelial cells with a great reduction in keratin content will respond to FN-matrix and grow in parallel array. The more interesting finding of this work, perhaps, is the adaption of added cells to the FN-matrix, resulting in a spatial organization of cells and fibronectin fibers as if the matrix were produced by the added cells. Acellular areas abundant in extracellular matrices are frequently detected in histological sections of various tissues. During metastasis when a single or a clump of tumor cells reaches a distant site, the ability of tumor cells to adapt to the new environment by adopting some of the extracellular matrices as a "surrogate matrix" may influence the probability of its survival. To assume a cell shape similar to that of the neighboring normal cells and to mimic the cellular organization similar to that of the surrounding tissue may be advantageous to the tumor cells at the initial encounter. Only after further cell proliferation leading to a critical mass may the need for "surrogate matrix" then subside.

During tissue regeneration, the influence of pre-existing extracellular matrices on the repopulating cells is another instance where the "surrogate matrix" may be useful. Although in certain cases the components of extracellular matrices may be removed or destroyed before regeneration, in other cases the remaining matrices (often as basement membranes) may serve as a guidance for the regenerating cells to restore the same pattern of cellular organization.

The phenomenon of using "surrogate matrix" by migrating cells may also exist during embryogenesis. Matrices can serve not only as the "temporary surrogate matrix" during contact guidance, such as in the course of the development of neural crest cells demonstrated recently by Thiery and his co-workers (24), but also as a "permanent surrogate matrix" for cells undergoing terminal differentiation. Moreover, since the orientation and organization of "surrogate matrix" were determined by previous cells residing in this environment, the transfer of certain information (cell shape, cellular organization etc.) from the matrix-forming cell type to the matrix-using cell type may be carried out spontaneously. If the matrix is of stable nature, information transfer may even be operable through a long time interval. The essence of time clock during embryogenesis may reside less on the so-called "intracellular program," and more on the cue encoded in the extracellular matrix, as repeatedly demonstrated by classical embryology.

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