Fibronectin Has a Dual Role in Locomotion and Anchorage of Primary Chick Fibroblasts and Can Promote Entry into the Division Cycle

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ABSTRACT Fibronectin (FN), which is already known to be a natural factor for fibroblast spreading on substrata, has now been shown to be essential for two distinct types of adhesion with different biological functions in chick heart fibroblasts, namely adhesion directed toward locomotion and toward stationary anchorage for growth. Manipulation of culture conditions and the use of antisera of differing specificities has demonstrated that both exogenous and cell-derived FN are important in each process. The organization of the fibronectin-containing matrix differs between the two states. Immunoelectron microscopy with a colloidal gold marker reveals the presence of small membrane-associated plaques of fibronectin in motile cells with associated submembranous specialization. A fibrillar matrix containing fibronectin is dominant in nonmotile, growing fibroblasts. The development of focal adhesions for stationary anchorage can be dramatically enhanced by addition of cell-derived FN at an appropriate stage, and this promotes entry into the growth cycle. New macromolecular synthesis in addition to FN is necessary for focal adhesion development but not for locomotion.

Fibronectin, a serum and cell-surface glycoprotein which promotes cell spreading and adhesion (30, 35, 50, also 28), has also been suggested to have a role in cell locomotion in vitro (2) and in vivo (12, 32). This would imply a duality of function in which the same glycoprotein would be involved both in labile adhesions to permit movement and in stable adhesions to develop microfilament bundles to anchor and flatten the cell body against the substratum. We have characterized a chick heart fibroblast system in which the two forms of adhesion are associated with distinct phenotypes and visibly different surface and internal structures (4, 10, 11). Cells migrating from suitably prepared explants pass first through a highly locomotory phase, characterized by interference reflection microscopy (14, 29) as having no focal adhesions and by transmission electron microscopy as having a fairly uniform, relatively unspecialized lower membrane contacting the substratum. Over 48 h the cells become stationary and develop numerous and substantial focal adhesions which appear as discrete black zones by interference reflection microscopy, with the lower membrane between the zones being lifted clear from the substratum. Both phenotypes are well flattened but differ in a number of further important respects—including the fact that motile cells are virtually growth-arrested, whereas stationary cells divide normally (4, 11).

We have used this system to investigate the possible duality of function of fibronectin and report that (*a*) fibronectin is indeed necessary for both movement and adhesion and that (*b*) adhesion for movement requires both exogenous and cell-produced fibronectin, even though the latter is expressed only in small amounts; adhesion for anchorage requires higher levels and/or a different molecular organization of cell-derived fibronectin. (*c*) Adhesion for anchorage, but not for movement, requires new macromolecules in addition to fibronectin, at least some of which are surface components. (*d*) Not only does the cell-derived fibronectin promote the development of focal adhesions when added at an appropriate stage, but it also then stimulates the cells to enter the growth cycle.

MATERIALS AND METHODS

Cell Preparation and Treatments

The methods for preparing explants of embryonic chick tissue have been fully described (11). Normally, the medium contained 10% fetal calf serum (FCS) in Modified Basal Eagle's Medium (MBEM, Flow Laboratories, Irvine, Scotland), and fibronectin was prepared from serum by the methods of Engvall and Ruoslahti (16). The resulting fibronectin-depleted serum was used in experiments and checked by immunoprecipitation for the lack of fibronectin. Fibronectin-depleted serum was also obtained from Dr. R. C. Hughes (National Institute for Medical Research, London) and used in the same experiments. Pretreatment of

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cover slips (described below) was for 2–3 h at room temperature before repeated washing in phosphate-buffered saline (PBS, Flow Laboratories) and assembly of the culture chambers. Cell migration from explants under various experimental conditions was scored on a five-point scale after 48 h. Migration under normal culture conditions was the standard for the maximum value, and the two criteria of the number of emergent cells together with the rate of emergence observed on time-lapse video recordings were used together as the basis for assessment. Cell-surface-derived fibronectin was prepared from confluent cultures of chick embryo fibroblasts by the methods of Yamada and Weston (49) but with an extra final dialysis for 16 h against serum-free MBEM and added to explant cultures at 50 μg/ml in complete medium. Bovine serum fibronectin was prepared as described above (16) and similarly added in complete medium. Chicken serum fibronectin (220 kdaltons) was a gift from Dr. A. Vaheri, University of Helsinki. This fibronectin was shown to be active in the promotion of cell spreading of secondary cultures in the absence of endogenous sources. Cycloheximide was obtained from Sigma Chemical Co. (Poole, Dorset, England) and tunicamycin was a gift from Dr. W. Cuthbertson (Glaxo Laboratories, Greenford, Middlesex, Eng.). Fluorescein isothiocyanate-conjugated (FITC) ricin (RCA 120; Miles Laboratories, London) was used at a dilution of 1:40.

Microscopy

Cells were examined live on a Leitz Ortholux II microscope fitted with epi-illumination and objectives for phase contrast, interference reflection, or fluorescence microscopy, as before (11).

Cells for immunoelectron microscopy were fixed in 3% paraformaldehyde in PBS (pH 7.4) at room temperature for 20 min, then washed and treated with 1% bovine serum albumin (BSA) for 15 min. A rabbit anti-chick fibronectin antiserum (47) was used at 1:50 dilution and incubated at 37°C for 45 min before washing. Cells were subsequently treated with a colloidal gold *Staphylococcus* protein A conjugate in PBS for 30 min at room temperature. After thorough washing in PBS the cells were postfixed in 3% glutaraldehyde (pH 7.4) in 0.1 M sodium cacodylate buffer for 15 min, followed by 1% osmium tetroxide (pH 7.4) in 0.1 M sodium cacodylate buffer for an additional 15 min. Before dehydration, cells were stained with 2% aqueous uranyl acetate. Dehydration, embedding, sectioning, and viewing procedures were as previously done (11). 5-nm colloidal gold particles were prepared by the white phosphorus/ether method (17) as modified by Romano et al. (42). Protein A (Pharmacia Fine Chemicals, Bromma, Sweden) was conjugated to the gold using the methods of Roth et al. (43).

Antibodies

Our experimental strategy required a series of antifibronectins of differing specificities. A rabbit anti-bovine antiserum was prepared as follows. Fresh bovine plasma was twice adsorbed with 10 mg/ml barium sulfate to remove vitamin K-dependent factors, adsorbed to DEAE-cellulose, then eluted with 0.5 M sodium chloride in 20 mM imidazole-HCl (IMH) at pH 7.5. Ammonium sulfate (25%) precipitation of the eluted proteins was followed by centrifugation. The precipitate was taken up in IMH at pH 7.5 and adsorbed onto epichlorohydrin, triethanolamine, and cellulose (ECTEOLA-cellulose). The bound proteins were eluted with 0.8 M sodium chloride in IMH at pH 7.0 and precipitated once more by ammonium sulfate (35%).

This precipitate was redissolved and applied to a gelatin-Sepharose column, washed with 0.5 M sodium chloride in IMH (pH 7.5), and eluted with 8 M urea in IMH at pH 7.5. The pure fibronectin was dialyzed overnight at 4°C against 0.4 M NaCl in buffer at pH 7.0, and injected into rabbits in complete Freund's adjuvant (47). Antiserum was used at a 1:50 dilution in serum-free medium (SFM) for live cells or in PBS for immunofluorescence microscopy.

Samples of the antiserum were adsorbed with chick plasma proteins (Flow Laboratories), after which the antiserum no longer yielded a precipitin line against chick serum or purified chick plasma fibronectin by double immunodiffusion tests, and had lost the ability to stain chick cell surface and intracellular fibronectin in cell cultures examined as previously (11) by indirect immunofluorescence microscopy. Cell-surface fibronectin on MDBK cells, a kidney epithelial line of bovine origin, stained positively with this adsorbed antiserum, however.

Rabbit anti-chick serum fibronectin was prepared and used as described previously (47). This antiserum failed to stain any mammalian cell-surface fibronectin including bovine on which it was tested by indirect immunofluorescence. Double immunodiffusion tests against bovine fibronectin or FCS were also negative. A rabbit anti-human fibronectin antiserum preadsorbed with fetal calf proteins was a gift from Dr. A. Vaheri. This antiserum was diluted 1:50 or 1:100 in SFM or complete medium for addition to living cells or in PBS for indirect immunofluorescence microscopy.

Indirect immunofluorescence microscopy was carried out as described previously (3, 47), with antisera against fibronectin or avian smooth muscle actin. In both cases the second antiserum, a goat anti-rabbit IgG conjugated to FITC, was diluted 1:50 before use.

Autoradiography

Explants were prepared as before (11), and after 18 or 64 h in culture the medium was aspirated and replaced with MBEM + 10% FCS containing one of the following labeled precursors: 0.3 μCi/ml [4,5-^{3}H]leucine (15.7 Ci/mmol), 0.1 μCi/ml [6-^{3}H]uridine (5 Ci/mmol), 2 μCi/ml D[1-^{3}H]glucosamine (4.1 Ci/mmol) or 6 μCi/ml D[1-^{3}H]mannose (5.1 Ci/mmol) (Radiochemical Centre, Amersham, Buckinghamshire, Eng.). For glucosamine autoradiography, experiments were carried out in serum (3 mg/ml) that had been dialyzed against distilled water and lyophilized. After 5-h incubation at 37°C the medium was removed and the cells were washed several times in complete medium followed by two washes in PBS and fixation in 3% glutaraldehyde in PBS. After further washes in PBS the cover slips were rinsed in distilled water before immersion in a solution containing equal volumes of 1% glycerol and K2 nuclear photographic emulsion (Ilford Ltd., Essex, Eng.). The emulsion was allowed to gel at 4°C, and, after drying at room temperature overnight, the cover slips were stored for 7 d. Processing was done for 3 min in D19 developer (Kodak) followed by 5-min immersion in 20% Hypam (Ilford). Mounted cover slips were examined and mean grain counts per cell were counted for 35 cells per cover slip. Each data point in Results represents the average grain count from four to eight cover slips.

For experiments on the effect of added cell-derived fibronectin on [^{3}H]thymidine uptake, the culture medium over 24-h-old explant cultures was supplemented either with SFM (control) or an equivalent volume of SFM containing cell-derived fibronectin to bring the final concentration in the medium to 60 μg/ml. Explants were further incubated for 16 h, after which the medium was replaced by MBEM + 10% FCS containing 0.5 μCi/ml [methyl-^{3}H]thymidine (5 Ci/mmol) (Amersham) for an additional 24 h incubation. Autoradiography processing was done as described above and from each cover slip, 350 cells were examined and the percentage having >10 grains over the nucleus was calculated. The data represent the mean of eight experiments.

TABLE I

Chick Heart Fibroblasts under Various Culture Conditions

	Incubation medium		
Cover slip treatment	10% FCS	10% FCS (Fn depleted)	SFM
10% FCS	+++++	+++++	++
10% FCS (Fn depleted)	+++++	+	+
SFM	—	—	0
10% FCS (Fn depleted) + 100 μg/ml fetal calf Fn	—	+++	—
70 μg/ml bovine plasma Fn	+++++	+++++	++

Migration of chick heart fibroblasts, 48 h after explanation, under various culture conditions (0, no migration; +++++, maximal migration; —, experiment not performed).

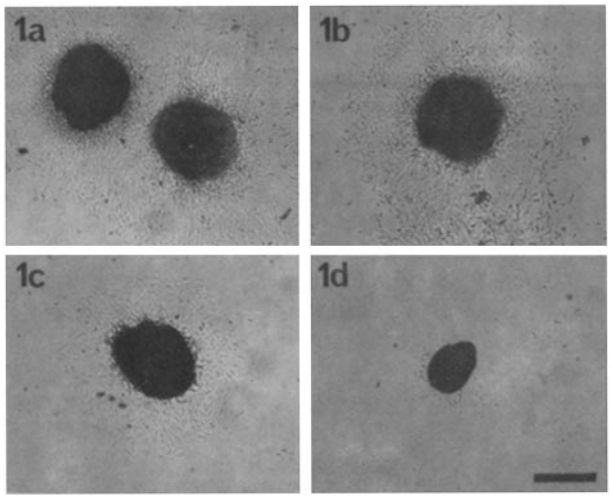


FIGURE 1 Low-power micrographs of embryonic chick fibroblasts, 48 h after explanation. *a*, control, MBEM + 10% FCS; *b*, bovine fibronectin-coated cover slip with MBEM + 10% fibronectin-depleted FCS; *c*, bovine fibronectin-coated cover slip in serum-free MBEM; *d*, serum-free MBEM. Bar, 500 μm. × 16.5.

RESULTS

Exogenous Fibronectin in Cell Locomotion

Fibroblast migration was assessed (as described in Materials and Methods) from explants incubated in media with or without exogenous fibronectin on substrata that had been pretreated or not with fibronectin. The results (Table I, Fig. 1) showed that migration is severely diminished when fibronectin is omitted from both the medium and the cover slip treatment but can be restored by pretreatment of the substratum with fibronectin, even in fibronectin-free medium. The requirement for substratum-bound fibronectin is further confirmed by the abolition of migration when fibronectin-coated cover slips are exposed to anti-bovine fibronectin. In addition to the requirement for fibronectin and other products of cellular origin for the steps in our sequence of cellular changes, it is clear that unknown factors in the serum are also important. This was shown by an experiment in which (on cover slips precoated with fibronectin) migration from the explant was considerably diminished in SFM as compared with medium containing

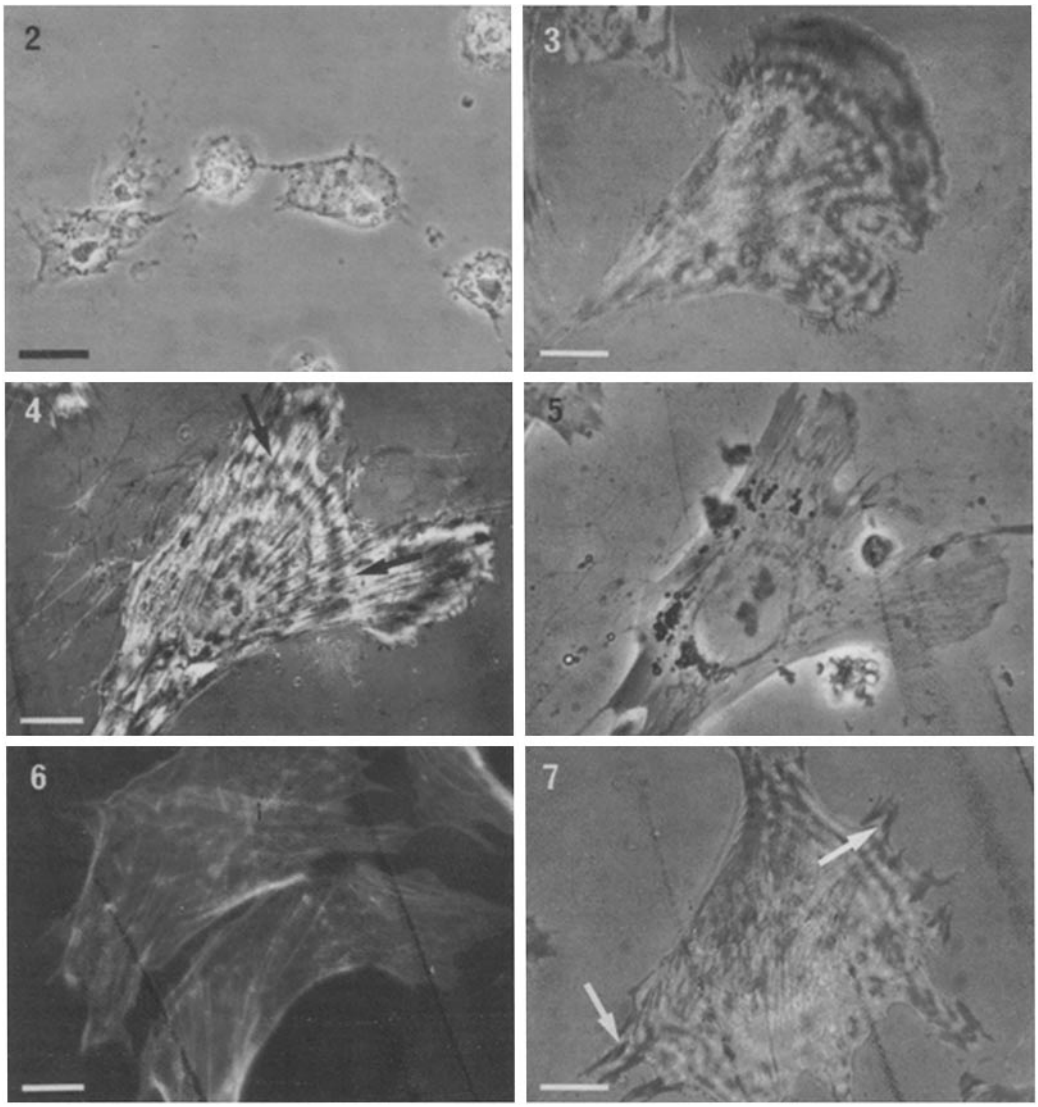


FIGURE 2 Motile fibroblasts rounded by the action of rabbit anti-bovine fibronectin antiserum. This antiserum binds to both serum and cell-derived fibronectins. Bar, 20 μm. × 460.

FIGURE 3 Early motile chick fibroblast as seen by interference reflection microscopy after addition of 50 μg/ml cell-surface fibronectin. Treated cells are morphologically indistinguishable from untreated cells. No focal adhesions are present. Bar, 10 μm. × 900.

FIGURES 4 and 5 Matching interference reflection and phase-contrast micrographs of an intermediate-stage chick fibroblast 2 h after addition of 50 μg/ml cell-surface fibronectin. Many focal adhesions (arrows) are present with associated microfilament bundles. Bar, 10 μm. × 800.

FIGURE 6 Fibroblast treated as in Figs. 4 and 5, stained by indirect immunofluorescence microscopy with antibodies directed against actin. Numerous microfilament bundles can be seen. Bar, 10 μm. × 800.

FIGURE 7 Interference reflection micrograph of a stationary chick fibroblast showing peripheral focal adhesions (arrows), unaffected by additional cell-surface fibronectin. Bar, 10 μm. × 900.

fibronectin-depleted serum (Table I, Fig. 1). Maximum mobility is seen in medium containing FCS. Serum factors additional to fibronectin therefore assist the migratory process. This is analogous to the effects that exist in cell spreading of fibroblast lines which have shown the need for serum factors in addition to fibronectin for the development of focal adhesions (47).

Responses to Antifibronectins of Differing Specificities

We used antisera of differing specificities to distinguish the roles of exogenous and endogenous fibronectins for each of the two phenotypes. An anti-bovine fibronectin serum known to cross-react with fibronectins from chick cells and FCS rounded the cells of both phenotypes (Fig. 2). Antiserum adsorbed with chick serum proteins, which was shown to remove also the cross-reaction with chick cell-derived fibronectin, rounded both phenotypes with the more rapid influence on the motile cells after 2–3 h incubation at 37°C. An anti-human fibronectin, preadsorbed with FCS proteins after which it retained the ability to decorate the extracellular matrix of chick fibroblasts, also rounded both phenotypes—with the quickest and most drastic effects being (as before) on motile cells. This antiserum had no effect after further absorption with chick serum proteins.

The process of cell rounding was similar for both motile and stationary cells. The first effects were seen at cell margins that were drawn inward, leaving short retraction fibrils attached to the substratum. In stationary cells many of these fibrils extended into areas where focal adhesions had been present, but for both phenotypes the experiments usually culminated in the detachment of retraction fibrils from the substratum to leave a fully rounded cell (Fig. 2). Since the early effects of antibodies on chick fibroblasts were to cause cell rounding without disruption of contacts to the substratum, we conclude that fibronectin has an important influence on cell shape and adhesion through interaction with regions of cell membrane, away from the focal adhesions, in addition to roles it might have at the actual adhesions themselves. In many respects this process was the same as adding a detachment agent such as trypsin and has been described for these cells previously (10). When cells of the locomotory phenotype became rounded in these experiments, migration was abolished. Control experiments with complement-inactivated antisera showed that these effects were not due to complement-mediated killing.

Responses to Elevation of Fibronectin Levels

Addition of chick cell-surface fibronectin (50 μg/ml) had no visible effect on the morphology of the early locomotory fibroblasts (Fig. 3), and time-lapse video photography showed that cell movement continued with no change of rate. In contrast,

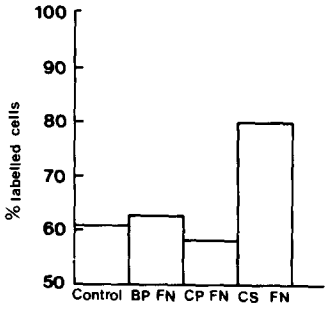
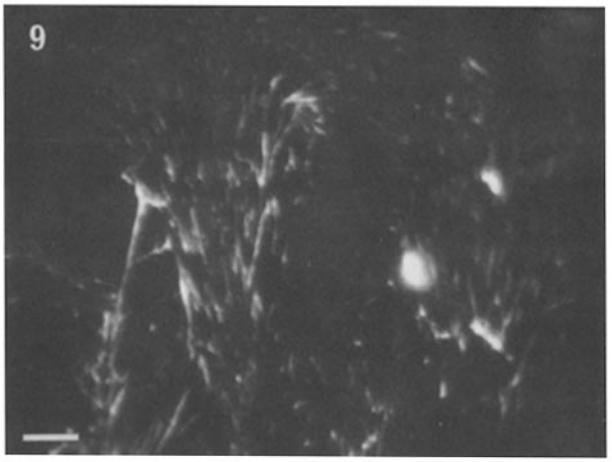


FIGURE 8 Effect of chick cell-surface fibronectin and plasma fibronectins on [^{3}H]thymidine uptake by primary chick heart fibroblasts. *BPFN*, bovine plasma Fn; *CPFN*, chick plasma Fn; *CSFN*, chick cell-surface Fn.



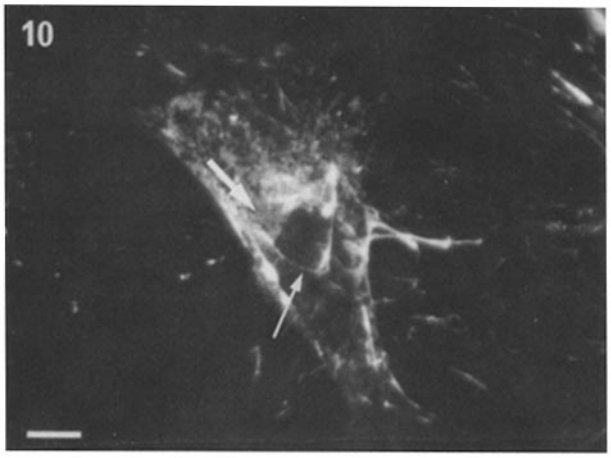


FIGURE 9 Indirect immunofluorescence micrograph of two migrating fibroblasts stained to show fine streaks of fibronectin on the upper cell surface. Bar, 10 μm. × 700.

FIGURE 10 Fibronectin staining of an acetone-extracted motile fibroblast showing intracellular staining (large arrow) and small fibers beneath the cell (small arrow). Bar, 10 μm. × 700.

cells beginning to undergo the phenotypic change as shown by development of focal contacts and filopodia (11) showed dramatic changes. The addition of cell-derived fibronectin caused large numbers of substantial and dense focal adhesions to appear within 2 h (Fig. 4) together with thick microfilament bundles which can be seen by interference reflection, phase contrast, and immunofluorescence microscopy (Figs. 4–6). These features in the altered cells were even more prominent than in the stationary phenotype to which the same cells would have progressed normally in culture (cf. Figs. 4 and 7). However, stationary cells which developed normally (with synthesis of their own copious deposits of associated fibronectin—see below) showed no further change when treated with additional chick fibronectin. Plasma fibronectin of bovine or chicken origin added to a concentration of 50 μg/ml had no visible influence on any of the cell types.

We reported previously that fibroblasts migrating from explants have a low growth rate and are mostly in G_1, in contrast to the stationary cells with focal adhesions which had a much elevated mitosis rate (11). To examine whether the acceleration of phenotypic change brought about by exogenous cell-derived fibronectin was associated with an earlier commitment to increased growth, we followed the process by autoradiography with [^{3}H]thymidine. Fig. 8 shows that where cell-derived fibro-

nectin was added to 24-h-old explants and replaced with [^{3}H]-thymidine at 40 h, there was a consequent increase in the number of cells entering S phase over the ensuing 24 h. Bovine and chicken plasma fibronectins were again without effect.

Expression of Endogenous Fibronectin

Earlier work (11) showed that the stationary phenotype expressed copious amounts of cell-derived fibronectin whereas the locomotory form expressed little that was visible by immunofluorescence. However, our new results (above) show that this small amount has a significant role in motility and therefore we have reexamined its distribution in more detail. In contrast to the stationary cells which express their fibronectin mostly as a component of a substantial fibrillar matrix, the locomotory cells showed only short, fine streaks on the upper surface at the light microscopy level, nearly always with the long axis parallel to the direction of locomotion (Fig. 9). Acetone extraction before immunofluorescent staining reveals fibronectin underneath the cells also (Fig. 10) and shows further that nearly all cells are producing fibronectin, as revealed by intracellular staining (Fig. 10).

Immunoelectron microscopy with a colloidal gold marker and an antiserum that only recognizes cell-derived fibronectin in this system confirms the expression of small amounts of fibronectin on the upper and lower surfaces of locomotory cells (Figs. 11 and 12): most of this appears "fuzzy" rather than fibrillar and is in small, distinct patches that are far smaller (up to 0.6 μm) than those expected of focal contacts or focal adhesions; they appear to be associated with some submembranous specialization including filamentous material (Fig. 12).

Protein and Glycoconjugate Production for Movement and Adhesion

When cycloheximide was added to the culture medium at 0.2 μg/ml from the time of explantation, cell migration occurred as usual but cells did not develop the usual compliment of focal contacts or focal adhesions (Figs. 13 and 14). A minority of the cells did develop small dark grey patches visible by interference reflection microscopy, but these did not possess the characteristics of focal adhesions that are assessed in living cells by their shape, dimensions, location, and image intensity in the interference reflection microscope. Addition of cell-derived fibronectin at 70 μg/ml failed to bring about the phenotypic conversion of these cells. Some fibronectin is still expressed in the presence of cycloheximide, presumably derived from an intracellular pool (not shown).

In the presence of tunicamycin at 0.2 μg/ml, the outwandering cells are diminished in number, somewhat less well spread than their counterparts in control cultures (Fig. 15), and again failed to develop focal adhesions (Fig. 16) although some cells

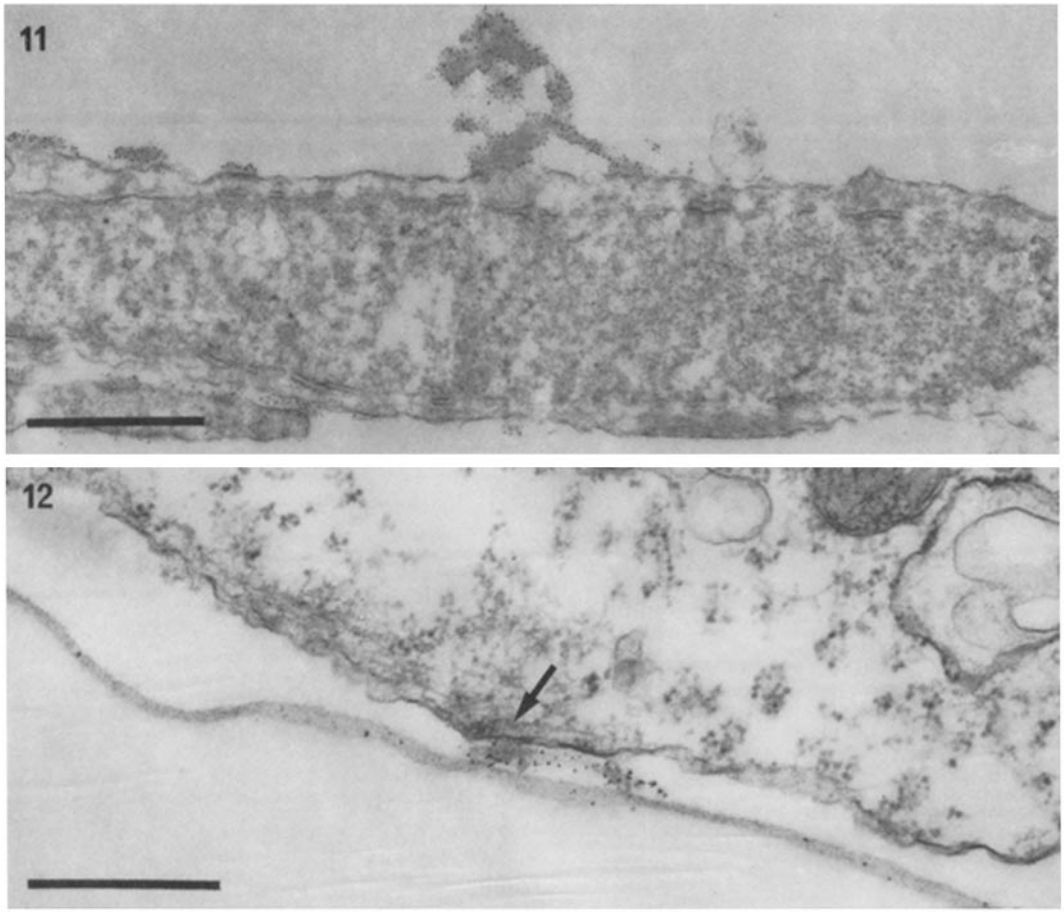


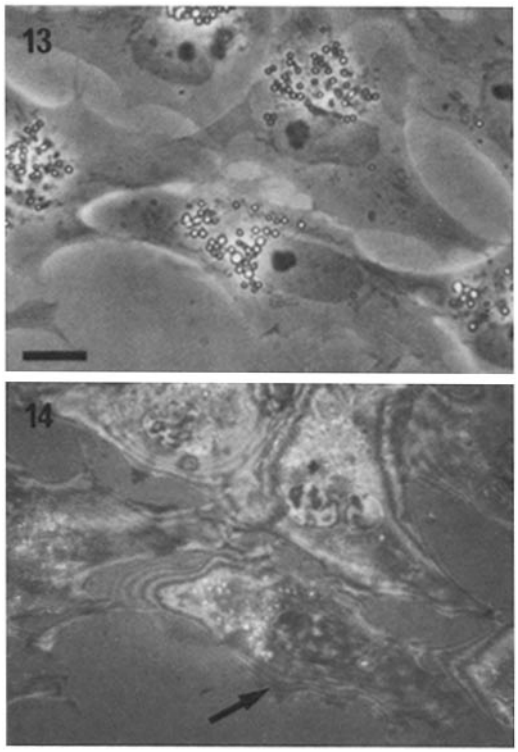
FIGURE 11 Immunoelectron micrograph with a colloidal gold marker of a motile fibroblast showing small cell-surface-associated patches of fibronectin on upper and lower surfaces. Bar, 1 μm. × 23,000.

FIGURE 12 A small amorphous patch of cell-surface fibronectin on the lower surface of a motile chick fibroblast, decorated by a colloidal gold marker. Note the associated submembranous plaque of filaments (arrow). Bar, 0.5 μm. × 50,000.

did form very transient dark grey streaks at their margins visible by interference reflection microscopy. Such structures may have been very small focal contacts. Fibronectin is expressed (shown by immunofluorescence) but at a low level (Fig. 17); some of this is stained by fluorescein-labeled ricin (Fig. 18), showing that either glycosylation is inhibited incompletely under our conditions or that early expression of fibronectin comes from a previously glycosylated intracellular pool (see above). The addition of cell-derived fibronectin to explants treated with tunicamycin is without effect at any stage, and focal adhesions do not form.

These experiments indicated that de novo synthesis of proteins and/or glycoproteins is required for the cells to convert to the stationary phenotype with fully developed focal adhesions. This cannot be solely a result of the absence of fibronectin, since this can be detected in the extracellular matrix in the presence of either drug and has no effect if exogenously added.

Further evidence that glycoconjugates have an important role in this phenotypic progression is that [^{3}H]glucosamine and [^{3}H]mannose incorporation are shown by autoradiography to be depressed over the first 24 h (the highly migratory phase) but then increase with the transition from motile to stationary state (Fig. 19). In contrast, synthesis of cellular RNA and synthesis of protein proceed at a fairly constant rate throughout the whole period, as measured by [^{3}H]uridine and [^{3}H]leucine uptake (Fig. 19). Virtually all the silver grains from [^{3}H]mannose autoradiography were found over the cells, whereas grains from [^{3}H]glucosamine labeling were scattered over cells and substratum. This confirms that, as expected from the well-established biosynthetic pathways (38, 41, 45), glucosamine is incorporated into both cell-surface and extracellular matrix components, including glycoproteins and glycosaminoglycans, whereas mannose is predominantly incorporated into cell surface glycoconjugates. Evidently, the incorporation of both classes of conjugates is reduced in early motile stages but later increases with progression through to the stationary phenotype.



FIGURES 13 and 14 Matching phase-contrast and interference reflection micrographs of chick fibroblasts after 48 h in culture in the presence of 0.2 μg/ml cycloheximide. No focal adhesions have formed but a few small dark areas are visible (Fig. 14) which do not have the characteristic form of focal adhesions (arrow). Bar, 10 μm. × 800.

DISCUSSION

In this paper we have shown that both adhesion for motility and adhesion for anchorage require fibronectin, but the form of the matrix and its interactions at the cell surface differs between these states. Furthermore, evidence is presented that the form of this extracellular matrix can greatly influence the behavior of chick fibroblasts, particularly with respect to growth. We have previously shown (11) with this system that growth rates are very low for motile cells, and this may have functional significance for a process that is analogous to a wound-healing response. Here we have shown that part of the influence on growth potential is derived from the matrix, which, if altered, can promote growth prematurely.

Motile adhesion and stationary fibroblast adhesion to substrata are distinct in terms of structure and function (4, 10, 11). In the first, much of the underside of the flattened cell is closely applied to the substratum and the adhesive bonds must be such that they can be made and broken for locomotion. The second type is more well known since it has been described for many primary cells and cell lines; adhesion is characterized by focal adhesions (1) which are associated at the inner face with actin bundles and other muscle proteins (3, 5, 7, 20, 24), and the adhesive bonds are formed by arrays of external macromolecules that show lateral periodicity (5). The precise role of fibronectin at focal adhesions is still controversial at present; we and some others (3, 28) have proposed that fibronectin mediates the binding to substratum at the actual site of focal adhesions, but another view (6, 9, 18) is that it functions in surrounding areas. Our experiments with selective antisera do show, however, that cell shape can be affected by antibodies directed at fibronectin which must be located away from the focal adhesions—because the membrane contact with substratum in the latter structures is not affected in the early stages of rounding. Whether this target fibronectin is on the upper surface, at close contacts (11, 18), elsewhere, or in some combination of these locations, is not known, but our evidence does suggest that fibronectin can bind to the cell membrane to control cell shape and adhesion without necessarily making tight bonds to the substratum.

That fibronectin is required for the labile bonds for cell movement follows from the observations that exogenous fibronectin is necessary as a substratum-bound species for movement to occur, and that antifibronectin causes the cells to round up and cease their controlled movement. Even though these cells express cell-surface fibronectin in small amounts only, this endogenous source is also necessary for the adhesion for locomotion, because an antiserum that does not cross-react with the exogenous fibronectin impairs adhesion and movement. We therefore conclude that this type of adhesion requires both endogenous and exogenous fibronectin.

Stable adhesions for anchorage also require cell-derived fibronectin because, at a certain stage in the phenotypic progression, formation of focal adhesions can be accelerated by addition of cell-derived fibronectin (thought not by serum-derived fibronectin of bovine or chicken origin to the same level), and furthermore, even when the adhesions are established, cell shape and maintenance of spread morphology can

also be impaired by antibodies specific for the cell-derived fibronectin. Addition of further quantities of cell-derived fibronectin to the moving phenotype had no influence on adhesion or movement, showing that the stationary cells require cell-derived fibronectin either at a higher concentration or in a different molecular organization.

The transition between phenotypes does not proceed suddenly but through recognizable intermediates (11). About 24 h after leaving the explant, many cells are still migrating, but time-lapse video photography with interference reflection optics shows the appearance of "focal contacts," which are distinct from focal adhesions in that they have a less dense image and are transient rather than stable (Fig. 4). It is at this stage (and not before or afterward) that cells respond to the addition of cell-derived fibronectin, suggesting that new macromolecules necessary for focal adhesions have now been synthesized or assembled, but not yet the appropriate level or form of fibronectin. Induction of the stationary phenotype with exogenous cell-derived fibronectin at this stage promotes focal adhesions and stress fibers which are considerably more developed than in the stationary cells that eventually appear naturally. We suggest that this exaggerated response arose because addition of fibronectin was not timed correctly with respect to other stages in the synthesis and assembly of external and internal structures. These results also imply that secretion of fibronectin in the appropriate organization or amount is necessary for natural development of focal adhesions and is coordinated in time with other biochemical events.

We have shown that addition of cell-derived (but not plasma) fibronectin to cells that have reached a transitional phase also promotes entry into the growth cycle. Such results demonstrate the importance of the constitution of the extracellular matrix in directing cellular behavior. Orly and Sato (34) have shown for some epithelial cells that the presence of extracellular fibronectin was required for cytokinesis, but in our case it is entry into S phase that is brought about by the addition of fibronectin because we have shown (11) that motile chick fibroblasts are in G_1. Simian virus (SV40)-transformed 3T3

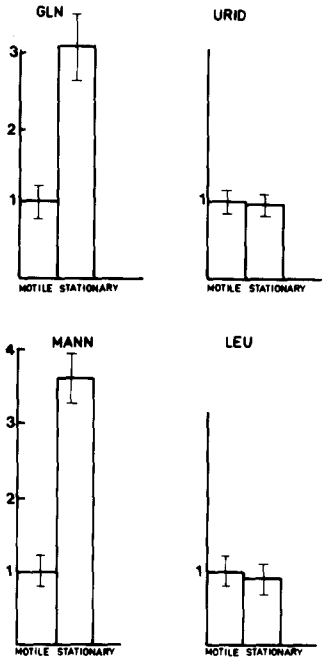
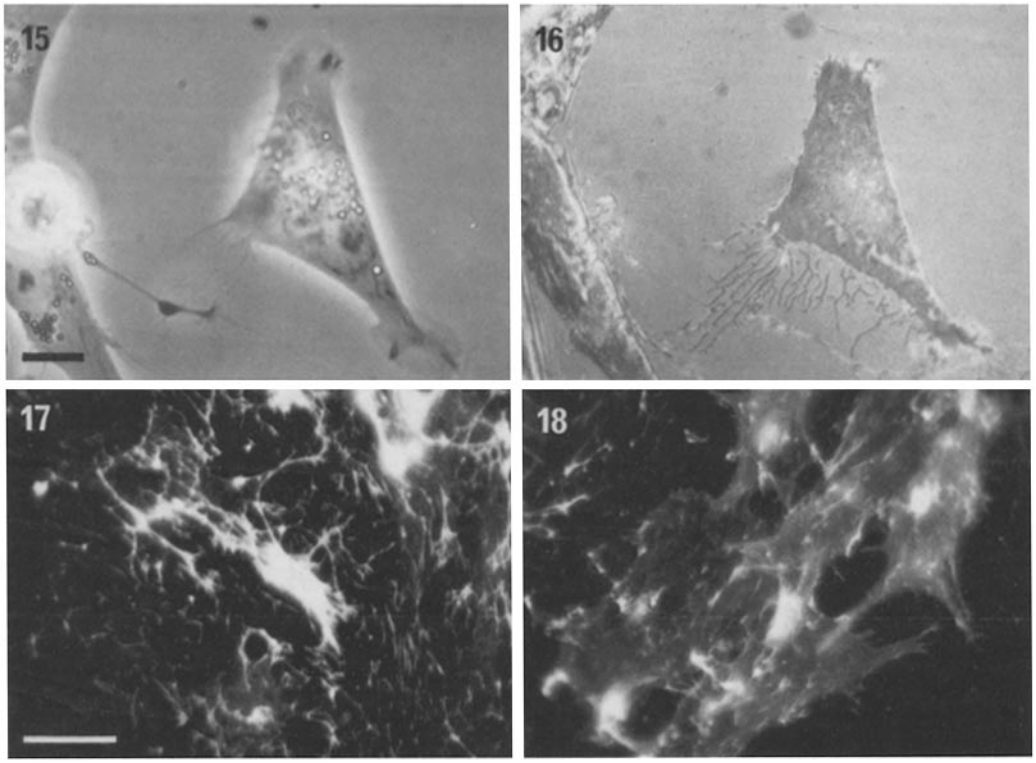


FIGURE 19 Incorporation of [^{3}H]glucosamine (*GLN*), [^{3}H]-uridine (*URID*), [^{3}H]mannose (*MANN*), and [^{3}H]leucine (*LEU*) by early motile and stationary chick heart fibroblasts.



FIGURES 15 and 16 Phase-contrast and interference reflection micrographs of chick fibroblasts 48 h after explantation in the presence of 0.2 μg/ml tunicamycin. No focal adhesions have formed but fine grey streaks are visible at the margin of the cell in Fig. 16. These may be very small focal contacts. Bar, 10 μm. × 800.

FIGURES 17 and 18 Tunicamycin-treated fibroblasts stained for fibronectin by indirect immunofluorescence and FITC-ricin, respectively. A comparison shows the persistence of some glycosylated fibronectin. Bar, 20 μm. × 600.

cells have also been demonstrated to require fibronectin-mediated attachment for growth (40), but here we show for nontransformed chick fibroblasts that growth is associated with a particular type of adhesion involving focal adhesion formation in which fibronectin plays a role. Our results are also highly compatible with recent reports (21, 44) where, for other cell types, biologically relevant extracellular matrices can crucially affect the capacity for growth in vitro.

Some insight into the changing nature of fibronectin secretion during the phenotypic conversion is gained by closer inspection of the membrane structures associated with the fibronectin deposits located on motile cells by immunoelectron microscopy (Figs. 11, 12). On the plasma membrane, these are small and the extracellular material is organized loosely, rather than being in the fibrillar form that is characteristic of stationary cells. This is similar to the results of Hedman et al. (25) which show that human fibroblasts have small patches of amorphous fibronectin-containing material on the cell membranes at early stages of culture, whereas in older, more dense cultures most staining was associated with larger patches and strands of extracellular matrix. Despite this lower level of extracellular organization in the motile cells, they show distinct hints of cytoplasmic density associated with the fibronectin-containing patches (Fig. 12), as noted also be Hedman et al. (25), which suggests an incipient plaque and hence some first beginnings of the assembly of a focal adhesion structure. These "primordial adhesions" occur only sparsely, and yet the intracellular staining in the same cells is as intense with antifibronectin (Fig. 10) as in stationary cells in which the extracellular deposits are copious. Whether the rate of synthesis of fibronectin is similar in the motile and stationary cells has not been ascertained, but much more is retained at the cell surface of the latter; this is the situation found when sparse and dense cultures or normal and transformed genotypes are compared in a similar way (19, 26, 27, 48, 50, 51).

Independent evidence that glycoconjugates are either synthesized and/or assembled at the cell surface at lower rates in the motile (compared with stationary) chick fibroblasts, was obtained by autoradiographic assay of the incorporation of [^{3}H]glucosamine and [^{3}H]mannose compared with [^{3}H]leucine (into proteins) and [^{3}H]uridine (into RNA). Whereas the accumulation of protein and RNA was similar at all stages of culture, the grain counts from both types of carbohydrate precursor were relatively depressed in the motile state (Fig. 19)—showing that the incorporation of both surface glycosaminoglycans and glycoproteins alters during the phenotypic progression.

The relevance of newly synthesized proteins and glycoconjugates to mechanisms of motility and anchorage in the chick fibroblast system was explored by blocking their synthesis, respectively, with cycloheximide and tunicamycin. Locomotion (which requires the formation of new labile adhesions) was not greatly affected by inhibition of protein and glycoprotein synthesis—whereas the development of focal adhesions was impaired with either blocking agent. The development of the stationary phenotype therefore requires the synthesis of new components at least some of which are glycosylated and presumably therefore destined for the external face of the plasma membrane. That this necessary synthesis is of components additional to fibronectin was shown by the following: (*a*) immunofluorescence evidence that fibronectin was still synthesized in the presence of tunicamycin even if this is underglycosylated, it would remain biologically active (33). (Even cells treated with cycloheximide still express small amounts of surface fibronectin, presumably from an intracellular pool present before drug treatment); (*b*) addition of cell-derived fibronectin did not reverse the effect of either blockage of synthesis.

One candidate for the new molecular species required for assembly of focal adhesions is the type of glycosylated component that is known to be associated with focal adhesions and neighboring areas of the cell underside and is a class of ricin receptors (3, 5). The elimination of such components in cell mutants causes cell adhesion to be impaired (15, 36). This would be consistent with the known affinity of ricin for terminal galactose residues linked to *N*-acetylglucosamine in the sequence that frequently terminates the type of chain whose addition to protein is blocked by tunicamycin (46).

Some of the new glycoprotein synthesis required for conversion to the stationary phenotype might be for receptors that bind directly to fibronectin, as in another system for which it has been proposed that interactions with fibronectin at the cell surface may be controlled by developmental regulation of fibronectin receptor(s) (23). This is, however, unlikely to represent the complete explanation because our experiments show that locomotory cells already have fibronectin receptors yet lack the ability to form focal adhesions. The results of our immunoelectron microscopy also demonstrate the association of discrete small patches of amorphous fibronectin-containing material with areas of upper and lower cell membrane. Possibly the receptors in this stage are present at a lower level and/or in a form that cannot undergo further clustering in the membrane because of different relationships to other membrane structures. An alternative or additional explanation might be that the tunicamycin block affects the assembly of other external species which consolidate focal adhesions, perhaps glycosaminoglycans, because tunicamycin is known to diminish the synthesis of sulfated glycoconjugates in chick fibroblasts (39) if not in other systems (8). There is evidence that glycosaminoglycans are involved in fibroblast adhesion to substratum (13) and are associated with fibronectin (37).

In summary, we have demonstrated that fibronectin is necessary for both motility and subsequent adhesion of primary chick fibroblasts. These results go some way to explaining the existing paradox arising from previous work which demonstrated that fibronectin was a causal agent in promoting adhesion for spreading where focal adhesion structures form (22, 47) and yet could also assist migration (2, 12, 32). It would appear that the state of the fibronectin and its association with the cell membrane are critical factors in this respect. Our motile cells, with labile adhesions, require fibronectin both on the cell surface and substratum and the observations by immunoelectron microscopy point to a nonfibrillar form of fibronectin. Other evidence (31) also points to distinct roles for substratum-bound fibronectin as well as a cell-surface form. In contrast, stable adhesion through focal adhesions with their associated cytoskeletal structures depends on the production of some new components over and above those present in the motile phenotype, namely protein and/or glycoprotein synthesis in addition to fibronectin. Crucially, the formation of focal adhesions and interaction with a particular matrix structure is necessary to enable growth to occur.

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