The E8 Subfragment of Laminin Promotes Locomotion of Myoblasts over Extracellular Matrix

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Abstract. The locomotion of murine myoblasts over the extracellular matrix components laminin and fibronectin was analyzed using quantitative videomicroscopy, and the organization of the cytoskeleton was observed in parallel immunofluorescence studies. Cells plated on the laminin-nidogen complex locomoted twice as fast as on laminin alone. The main form of translocation on laminin was a jerky cycle of prolonged lamellipod extension followed by rapid ($\sim$200–<500 μm h^{-1}) movement of the cell body into the extended lamellipod. The locomotion-stimulating activity of laminin resides in the elastase digestion fragment E8, part of the laminin long arm, while the El-4 fragment containing the three short arms is inactive.

Myoblasts moved poorly over fibronectin irrespective of whether high, intermediate, or low coating concentrations were used ($\sim$5,000–$\sim$10 fmol cm^{-2}). In contrast, the locomotory responses both to laminin and to E8 peaked sharply at coating concentrations $\sim$20–50 fmol cm^{-2} and decreased at higher concentrations. This response corresponds to that expected for a haptotactic stimulant. When cells locomoted over a mixed substrate of laminin and fibronectin, the fibronectin effects appeared to predominate.

The cytoskeleton has been implicated in many cellular motile processes. Within 6 h on fibronectin many cells expressed vinculin-containing focal contacts, elaborated stress fibers and had periodically organized α actinin, whereas on laminin, most cells showed diffuse vinculin and α actinin and a fine meshlike actin cytoskeleton. We conclude that the poor locomotion of cells over fibronectin is because of the cytoskeletal stabilization it induces.

MANY populations of embryonal cells, for example skeletal muscle myoblasts, migrate through a dense meshwork of extracellular matrix (ECM)[1] molecules to their developmental targets. Such specific cellular interactions with the ECM can heavily influence development (for reviews, see Hay, 1984). We have been interested in the behavior of skeletal muscle myoblasts in response to fibronectin, a component of the interstitial matrix, and laminin, found in basement membranes. Skeletal myoblasts migrate during development from the somitic myotome to the peripheral mesenchyme to form myotubes (Chevallier, 1979; Christ et al., 1983). During muscle repair, quiescent muscle "satellite" cells are activated to reiterate within the residual muscle basement membrane a migration and differentiation process (Vracko and Benditt, 1974; Cossu et al., 1980; Bischoff, 1986).

In vitro, the biochemistry and biology of myoblast differentiation is similar to that in vivo. There is an absolute requirement for ECM molecules as substrates and cytodifferentiation is profoundly affected by the nature of the molecules. Both laminin (LN) and fibronectin (FN) stimulate murine myoblasts and the satellite cell line MM14dy to attach (von der Mark and Kühl, 1985; Kühl et al., 1986; Foster et al., 1987; Goodman et al., 1987; Öcalan et al., 1988), but only LN will accelerate proliferation (Foster et al., 1987; Öcalan et al., 1988) and differentiation into nonreplicating myotubes (Kühl et al., 1986; Foster et al., 1987; Risse et al., 1987; von der Mark and Öcalan, 1989), while FN retards it (Podelski et al., 1979). The major myoblast binding site on laminin lies within the E8 subfragment, the lower 35 nm of the long arm (Goodman et al., 1987; Dillner et al., 1988).

The many specific effects of LN and FN on myoblast behavior suggest that they are recognized by specific receptors at the cell surface. Such recognition might affect embryonal patterning and development in vivo (Jaffredo et al., 1988), and might modulate myoblast migration and cytodifferentiation in vitro (Foster et al., 1987; Menko and Boettiger, 1987; Öcalan et al., 1988). Furthermore, the ECM can be connected over specific cell surface receptors (Hynes, 1987) to the cytoskeleton, a complex of intracellular structures deeply implicated in locomotory phenomena (Horwitz et al., 1986; Burridge et al., 1987).

Thus the questions arose, do different matrix components

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; LN, laminin; FN, fibronectin; AMP-CP, α,β methylene adenosine diphosphate.

initiate different locomotions? By what mechanisms do cells locomote over matrix components, and what role does the cytoskeleton play in the system? We therefore investigated whether specific matrix components could influence myoblast locomotion in vitro to understand how development and repair processes might be controlled. We examined the effects of laminin and fibronectin on locomotion using analytical video microscopy and on the cytoskeleton using immunofluorescence microscopy. In particular we asked which domains of LN trigger locomotion and what effects does the matrix density have on cell behavior.

We report here that LN activates myoblast locomotion in a characteristic narrow concentration range while FN does not. The elastase subfragment E8 (Edgar et al., 1984; Aumailley et al., 1987; Goodman et al., 1987; Goodman et al., 1989*a*) alone can fully activate myoblast locomotion, and does so with a very similar concentration dependence to whole LN. Characteristic and substrate dependent redistributions of cytoskeletal elements accompany the activation and repression of locomotion. We conclude that the effects of laminin are highly specific and may thus be significant during development and repair processes.

Materials and Methods

Cell culture methods, attachment and stripe locomotion assays (Kühl et al., 1986; Goodman and Newgreen, 1985; Goodman et al., 1987; Öcalan et al., 1988), the preparation of LN and LN-nidogen complexes (Paulsson et al., 1987; Goodman et al., 1987) and the preparation of the E8 subfragment (Edgar et al., 1984; Goodman et al., 1987) have been described in detail elsewhere. The laminin-nidogen complex is thought to be a more native form of laminin, and is extracted under physiological salt conditions using Ca^{2+} chelators from the Engelbreth-Holm-Swarm tumor (Paulsson et al., 1987). The E8 subfragment lacks the nidogen binding site.

MM14dy is a differentiation competent murine myoblast satellite cell line that attaches both to LN and FN substrates (Öcalan et al., 1988). The "stripe" assay employs adjacent parallel tracks of different proteins to compare the relative locomotion of cells (Newgreen, 1984; Goodman and Newgreen, 1985).

All tissue culture reagents were obtained from Gibco Laboratories (Grand Island, NY)/Bethesda Research Laboratories (Gaithersburg, MD), chemicals from Merck (Darmstadt), and other proteins from Sigma Chemical Co. (St. Louis). The quantities of proteins adsorbed onto the substrates were measured by their trace iodination and substrate coating as for locomotion assays, followed by gamma-counting of the adsorbed material. LN, E8, and FN were assumed to have relative molecular weights of 900,000, 280,000, and 480,000, respectively (Goodman et al., 1987).

Cell Locomotion Assays

Substrate Preparation. Proteins were routinely tested for activity in adhesion assays (Goodman et al., 1987; Öcalan et al., 1988). For video microscopy, 25 cm^2 flasks (3013E; Falcon Labware, Oxnard, CA) were coated by incubation, usually for 1 h at 37°C, with matrix protein diluted in 5 ml of Ca^{2+}, Mg^{2+}-free PBS (137 mM NaCl, 2.7 mM KCl, 1.45 mM KH_2PO_4, 8.0 mM $Na_2HPO_4.2H_2O$; pH 7.4), washed twice with PBS and residual protein binding sites on the flask blocked for at least 12 h at 4°C with 20 mg ml^{-1} heat-treated (80°C, 20 min) BSA (A7030; Sigma Chemical Co.). For concentration dependency experiments, the coating incubations were continued overnight at 4°C.

After washing with PBS, 5 ml of medium (unless otherwise stated, MM14 medium) were added (F10C containing 15% horse serum, 5% FCS, and 1% chick embryo extract (Öcalan et al., 1988)) and the flasks allowed to equilibrate in a 7.5% CO_2, 92.5% air atmosphere at 37°C for 1 h. Stripping fibronectin out of the sera (Engvall and Ruoslathi, 1977) had no effect on the subsequent cell locomotion (not shown).

When soluble reagents were tested, a culture was filmed as control for ~12 h, the medium removed, prewarmed pregassed medium containing the reagent added, and the flask returned to the microscope. Additions of the LN B1 chain peptide YIGSR (Graf et al., 1987) to a final concentration of 400 $\mu g\ ml^{-1}$ and the 5'-nucleotidase inhibitor α,β methylene adenosine diphosphate (AMP-CP) (Risse et al., 1987) to a final concentration of 60 $\mu g\ ml^{-1}$ were made by sterile addition of ~100 μl concentrated reagent through a syringe port welded into the side of the flask.

For heparinase treatment, substrates after coating and blocking were incubated for ~16 h at 37°C with heparinase from *Flavobacterium heparinum* (50 IU ml^{-1} in PBS; H7505; Sigma Chemical Co.), washed and used in video-locomotion assays.

Cell Preparation. MM14dy cells were routinely cultured in proliferative preterminally differentiated form at low density ($<1 \times 10^5$ cells/10-cm plate) in MM14 medium and prepared for assays as previously described for attachment and ligand binding studies (Goodman et al., 1987; Goodman et al., 1989*a*; Öcalan et al., 1988). For video microscopy, cells were seeded at 2×10^3 cells cm^{-2} in MM14 medium on coated flasks, allowed to equilibrate for 1 h at 37°C, and transferred to a microscope (ICM-405; Carl Zeiss, Inc., Oberkochen, FRG) thermostated to 37°C.

Video Microscopy and Analysis. The video-microscopy system has been described in detail elsewhere (Goodman et al., 1989*a*). Briefly, the cultures were recorded using low levels of illumination and a time lapse video recorder triggered from an external timer and fitted with a day-date generator. Recordings were usually continued for ≥12 h. The resulting films were projected onto a digitizing tablet and cell paths traced with the cursor, and analyzed under the control of a PASCAL program, "RUNA" (Goodman, unpublished observations), running on an IBM AT-compatible microcomputer. Cells were followed by visually judging and tracking the centre of the cell body (taken as the position of the cell nucleus when visible). The digitizer was set in stream mode; it reported the position of the cursor to ± 0.025 mm every 0.01 s. As each X-Y pair was accepted, the time was read from the internal clock of the computer and the data stored to give an itinerary for each cell.

RUNA corrected the cell itinerary for time lapse and magnification and for gating times and distances; a point was only considered as a movement from the previous point if it lay more than the gating distance away and took more than the gating time to get there. Thus, the data was filtered to the required level of sensitivity, and errors resulting from digitizer sampling and operator response time were minimized (see below; Profitt and Rosen 1979; Cornelisse and van der Berg, 1984; Heilbronner, 1988). RUNA calculates the distance and the time between successive points to generate "steps." It links the steps into a path from which it derives the speed of locomotion, the magnitude of the mean velocity of locomotion (in this work abbreviated as "velocity" of locomotion), the distance from the origin, and the local velocity between points. These values are defined below.

Cells tracks include cell–cell collisions. Cells plated at the standard cell density of 2×10^3 cells cm^{-2} moving on LN at a mean speed of ~70 $\mu m\ h^{-1}$ had a mean time between collision of 3.4 ± 2.2 h (n = 47).

We noted that the cells migrated in a very irregular manner (cf., Fig. 4 and Results). We therefore used a "speed along the path" algorithm, where we defined:

speed of locomotion = $\Sigma d/\Sigma t$
local velocity (along the step) = d/t
velocity of locomotion = $p/\Sigma t$
where:
d = The distance covered in a step (μm).
t = The time taken for a step (min).
p = The distance of the cell from its start position (μm).
Σt = The summed step times at the current cell position [min].
Σd = The summed step distances at time Σt (μm).

This algorithm was used because with uneven locomotion "speed and persistence" analyses are not reliable (Dunn, 1983; Wilkinson et al., 1984; Dow et al., 1987; Dunn and Brown, 1987). The value of Σt was constant (5 h) for the data tabulated in this study.

Graphic Displays

For ease of comparison, normalized "wind-rose" plots are usually shown; the starting positions of each cell have been X-Y translated so that it starts moving from (0,0) in the coordinate system (Fisher et al., 1989). This allows differences in population behavior to be readily seen. All graphics shown here are directly comparable; they have been normalized to show on axes 600-μm-wide, 5-h runs for 15 randomly selected cells from the filmed population (usually 20–40 cells/experiment). Rare changes to this format are noted in figure legends.

Estimation of Measurement Errors

Errors in the measured speeds and distances can derive from four sources:

distortion in the optical system, digitizing phenomena, operator errors, and resolution problems.

The linear distortion in the optical and recording system was <5% as measured by recording of a precision rectangular grid pattern (a hemocytometer). Many factors can affect digitizer measurements of paths (Cornelisse and van der Berg, 1984; Heilbronner, 1988). Therefore, to estimate digitizing accuracy and reproducibility, a single cell path was repeatedly traced ($n = 9$). When the paths were overlaid, the greatest track width was ~25 μm; thus, the uncertainty in cell position in both X and Y coordinates was approximately ±15 μm; i.e., <5% of the TV frame width (~500 μm) and the derived run time (9.74 ± 0.46 h), path length (562 ± 13 μm), and speed (56 ± 2.5 μm h^{-1}) also showed only low errors (95% confidence levels; $n = 9$).

Errors can also arise from parallax and user reaction time. As the digitizer was ~1 cm from the monitor screen, a 5% parallax error required a 30-cm eye movement. At the time compression we used, ~750-fold, an hour passes in ~5 s. Assuming a reaction time of 0.2 s, the lowest meaningful time gate is 3 min (750 × 0.2 s). For a cell moving at maximum velocity (~500 μm h^{-1}), this translates to a spatial error of ~12 μm, and at average velocity ~2 μm, well within the other system errors.

The "true" path of a locomoting cell probably has fractal dimensions. Its length thus depends on the length of the "ruler" used to measure it (Mandelbröt, 1983). However, using a defined ruler the relative speeds and velocities of different populations of cells, photographed under similar conditions, are comparable. Our ruler, the gating settings of the digitizer, and the RUNA program, were fixed at ~1 μm for the digitizer and 5 μm for RUNA in this study, settings that minimize the errors incurred in digitizer tracking of paths (Profitt and Rosen, 1979). The time gate was held at 5 min throughout the study.

In summary, we estimate that results derived with the system will routinely fall within ±10% of the "true" values for the speed and distance covered of moving cells.

Immunofluorescence

Multispot glass slides (Flow labs: ES-418-05) were cleaned by standing for 1 h in 70% ethanol, 2 h in 2 M HCl, and 2 h in 2 M NaOH, rinsed several times with water and autoclaved at 120°C. After coating with 5 μg ml^{-1} FN or LN in PBS and blocking as described above for video-microscopy, 2.5×10^3 cells/point were plated in 20 μl MM14 medium and incubated for various times at 37°C in 7.5% CO_2, 92.5% air atmosphere before preparation for immunofluorescence (Small et al., 1986). Cells were washed at 0°C with 2-(*N*-morpholino)ethanesulfonic acid-(MES) buffer (137 mM NaCl, 10 mM $MgCl_2$, 2 mM EGTA, 5.5 mM glucose, 10 mM MES; pH 6.1 at 20°C), permeabilized for 5 min at 0°C with MES-buffer containing 0.15% Triton-X 100, washed, and fixed for 20 min at room temperature with 1% paraformaldehyde in MES-buffer. After a PBS rinse, and 5-min treatment with freshly dissolved $NaBH_4$ (1 mg ml^{-1} in PBS), the cells were washed in PBS and monoclonal anti-vinculin or anti-α-actinin antibodies (undiluted hybridoma supernatants; the generous gift of Dr. B. Geiger, Rheovot) were applied (1 h at 37°C). After 3 washes for 3 min in PBS, FITC-conjugated goat anti-mouse IgG (Boehringer-Mannheim Biochemicals, Weizmann Institute, Mannheim, FRG) diluted 1:30 in 1% normal goat serum was added, together with 1 μg ml^{-1} rhodamine-phalloidin. After 1 h at 37°C, the preparations were again washed in PBS and mounted in 10% Moviol 4-88 (Hoechst), 25% glycerol, 100 mM Tris-HCl, pH 8.5, and viewed on a diaplan microscope equipped for epifluorescence (E. Leitz, Rockleigh, NJ) and photographed (TMAX film, 400 ASA; Eastman Kodak Co., Rochester, NY) using similar exposure and printing times and conditions.

Results

The E8 Fragment of LN Stimulates Locomotion of MM14dy

To assess whether the attachment and migration-promoting activities of LN reside in the same domain (Goodman et al., 1987, 1989*a*,*b*; Öcalan et al., 1988), we allowed MM14dy myoblasts to migrate over substrates consisting either of LN, or the LN fragments E8 and E1-4 (Fig. 1). MM14dy show highly reproducible behavior with no obvious differences to primary mouse myoblasts and are more consistent, more uniform, and easier to use than primary cultures. MM14dy moved at a similar apparent rate onto both E8 and LN substrates (Fig. 1 *C*), but not onto FN (Fig. 1 *A*). In marked contrast, cells offered the choice between LN and E1-4 moved rapidly away on LN stripes, but showed minimal migration on E1-4 stripes (Fig. 1 *B*). Movement over E8 was also vigorous compared with FN (Fig. 1 *D*) or with the blocking reagent, BSA (Fig. 1 *E*).

To quantitatively analyze the cell locomotion, we employed time-lapse video microscopy. Typical path analyses from individual experiments are shown in Fig. 2. Cells migrated rapidly over LN and E8 substrates but not over FN. The results observed on LN, FN, and E8 are summarized in Fig. 3 and Table I and support the qualitative results of the stripe assay. The speed of locomotion over E8 and LN was similar and ~3 times that over FN. On the LN-nidogen complex, the speed was ~7 times that on FN. Thus, both LN and the E8 fragment appear to lack a locomotion-promoting site present in LN-nidogen. The experimental data from E8 and LN appear to form a clustered group, while the FN and the LN-nidogen results are clearly independent.

Myoblast Locomotion over LN but not over FN Involves Cyclical Polarization and Depolarization

We wondered whether there was a basic difference in the mechanism of locomotion on LN and FN, and examination of the video films suggested that there was. There were two main motile forms that cells used to move over LN, LN-nidogen, and E8. The most common was a repetitive cycle of pseudopod extension where the cell body remained stable, followed by its rapid release toward the pseudopod (essentially rounding up toward the pseudopod). Alternatively, another pseudopod could be extended, often diametrically opposed to the one already extended. These movements produce the bipolarized spindle forms characteristic for myoblasts on LN (e.g., Kühl et al., 1986). Bilaterally polarized cells were static, or moved, apparently as one pseudopod released from the substrate, with rapid translocation of the cell body (250–>500 μm h^{-1}; Fig. 4) toward the other pseudopod, to give a rounded cell awaiting another cycle of polarization. Most of the cells were continually cycling through these changes. This led to a rather jerky progression of the cell body over the substrate (Fig. 4). Short periods of very rapid translocation of the cell body were interspersed with pauses in which pseudopod extension occurred. In the other motile form, rounded cells polarized by extending a single short pseudopod bearing vigorous ruffling leading lamellae, which the cell body followed over the substrate. The motion resembled that of neutrophils (Zigmond, 1977; Lackie, 1986).

Both forms of motion were sometimes interrupted by withdrawal of the pseudopod, cell rounding and subsequent initiation of the polarization cycle. Alternatively, pseudopods could split into two and, rather like a breast stroke swimmer pulling back the arms, these would move down the sides of the cell until more or less at right angles to the original direction of locomotion and diametrically opposed. Cells sometimes made a long ruffle across the opening pseudopods and began crablike motion.

On FN the behavior was fundamentally different. The slow cell motion was much more uniform and more fibroblast-like (Fig. 4). The cell body elaborated a short leading lamella

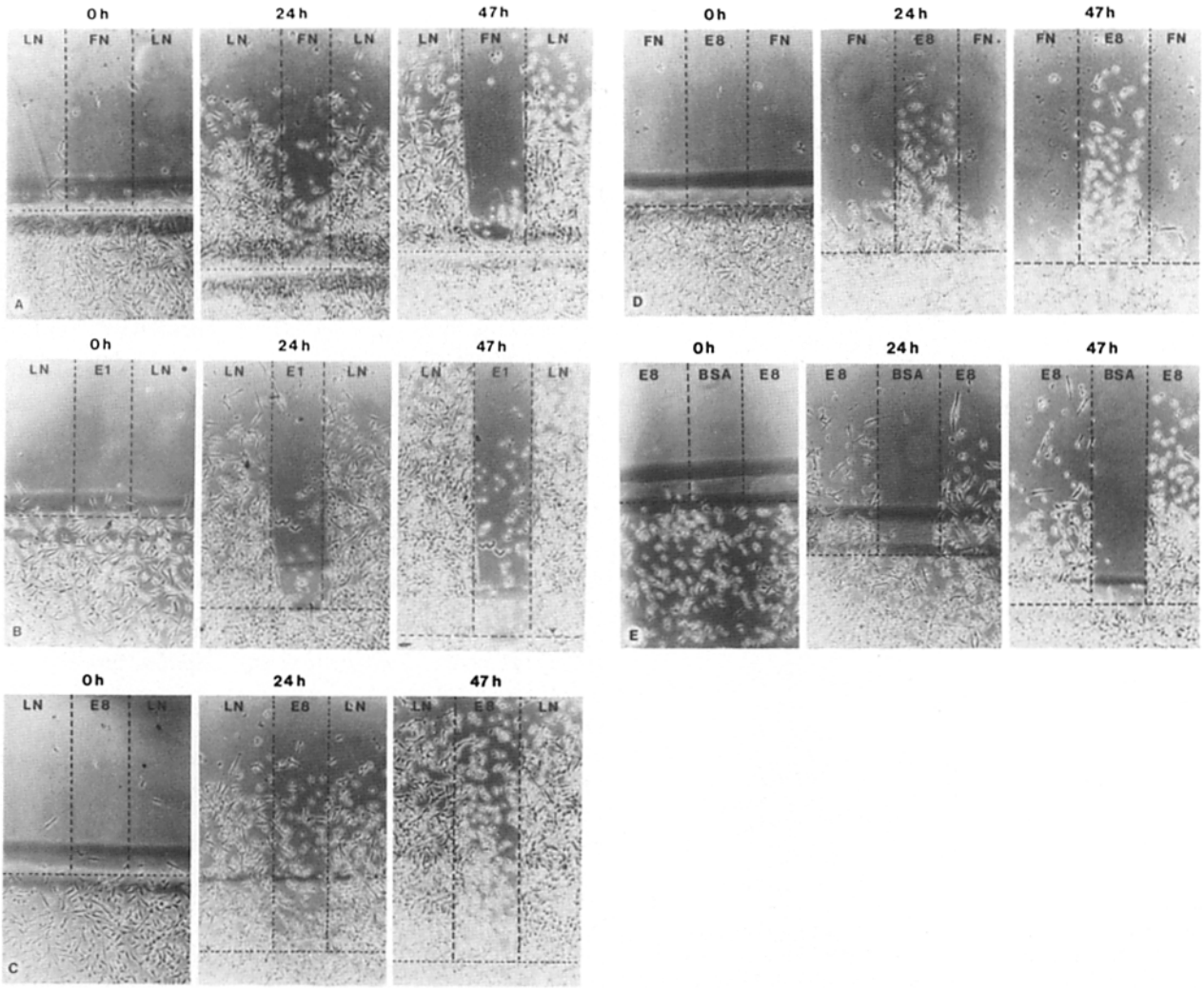


Figure 1. The locomotion of mouse myoblasts over LN subfragments. Stripe assays were performed as described in detail in Materials and Methods. Parallel stripes of each of two different proteins were adsorbed onto the substrates and MM14dy cells plated at similar densities at one end of the tracks and allowed to migrate. The three panels in each plate show the state of the assay at 0, 24, and 47 h after the start. (*A*) LN versus FN. (*B*) LN versus E1-4 subfragment. (*C*) LN versus E8 subfragment. (*D*) FN versus E8 subfragment. (*E*) E8 subfragment versus BSA control.

that they followed slowly over the substrate. Bipolar cells were static, there was neither release of trailing lamellae nor rapid movement of the cell bodies. However, the cells were not paralyzed; they bore actively ruffling leading lamellae, and occasionally extended a pseudopod. When cells cultured on FN were transferred to LN, they immediately commenced locomoting. While on transfer from LN to FN, cells became static.

The Failure of MM14dy to Move over FN Is not a Result of Gross Adhesive Differences

We wanted to understand the basis for the inability of cells to move over FN and their ability to move over LN. An obvious possibility was that cells immobile on FN were too strongly attached. To test this hypothesis, video-microscopy locomotion assays were performed using substrates coated with protein concentrations ranging from those where cells were very weakly adherent to those at least an order of magnitude above the point of maximum cell attachment (Goodman et al., 1987; Öcalan et al., 1988). The results are shown in Fig. 5.

For E8 and LN, there were coincident peaks of locomotion stimulatory activity corresponding to a coating concentration of ~1 μg ml^{-1} (Fig. 5 *a*). Both LN and E8 produced rather similar stimulation of locomotion. Maximum speed was ~70 μm h^{-1}. At both lower and higher coating concentrations, the cell migration decreased and reached the low values seen for cells on FN, which were uniformly sedate over the concentration range (~10 μm h^{-1}). Even when we used FN coating concentrations where cells were only weakly attached, there was also no movement.

Recalculating the data in terms of moles of matrix molecule on the substrate (Fig. 5 *b*) revealed that LN and E8 stimulate maximal locomotion within a very narrow coating range, at densities between 20 and 50 fmol cm^{-2}; by a density of ~100 fmol cm^{-2}, the locomotion is retarded. By contrast, a range of FN coating densities between ~10–~5,000 fmol cm^{-2} did not stimulate myoblast locomotion. Thus, a wide range of FN concentration affects locomotion and adhesion only slightly, while changes in concentration of either LN or E8 drastically alter the motile but not the adhesive response of the cells.

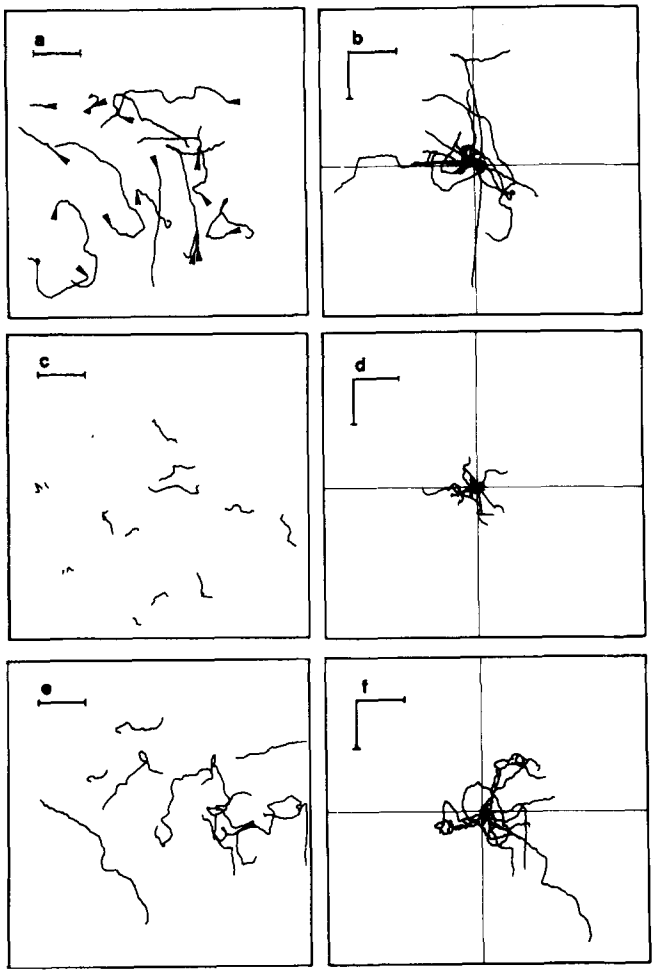


Figure 2. Video microscopy of myoblasts locomoting over LN, FN, and E8 subfragment. Video microscopy of MM14dy cells migrating over (*a* and *b*) LN; (*c*, and *d*) FN; and (*e* and *f*) the E8 subfragment of LN. In *a*, *c*, and *e*, the paths of the cells as they appeared on the screen are shown, normalized for 15 cells over 5 h. In *b*, *c*, and *f*, the same data has been converted to wind-rose displays, where all cells start from the same point, allowing differences between the runs to be more readily grasped. The starting position and direction of motion of the cells on LN in (*a*) has been marked with an arrowhead. Clearly, cells on FN migrate more slowly than on LN or E8. Bars, 100 μM. The techniques are as described in detail in Materials and Methods.

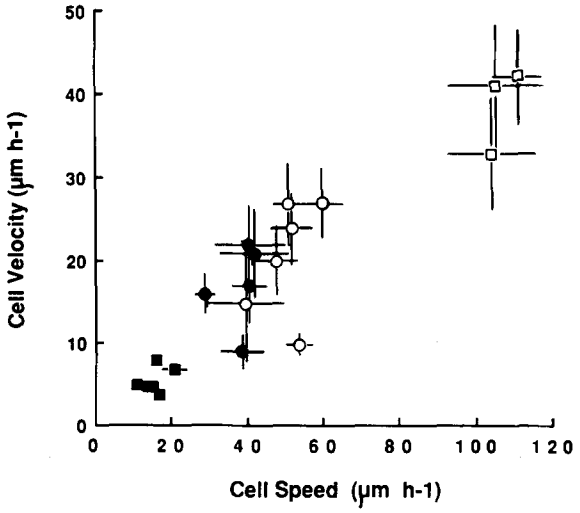


Figure 3. Myoblast locomotion over LN, FN, E8, and the LN-nidogen complex. Each point represents the pooled behavior of 10–30 cells from an individual video analysis as described in detail in Materials and Methods locomoting over LN (*open circles*), FN (*solid squares*), E8 subfragment (*solid circles*), and the intact LN-nidogen complex (*open squares*). Error bars, 1 SD (see also Table I).

shown). Thus, on FN substrates the cytoskeleton tended to the organized form seen in immobile cells, while on LN the less focally ordered form characteristic of motile cells was found.

When FN Is Mixed into LN Substrates MM14dy Locomotion Is Retarded

To test whether the effect of the ECM components on cell behavior was mutually independent, or if one dominated over the other, we studied the effect of using mixtures of proteins on the substrate. LN-nidogen complex stimulated cells to locomote twice as fast as LN, or E8 alone (Table I). An equimolar mixture of FN/LN-nidogen produced a 30–40% reduction in speed compared to pure LN-nidogen (Table II). A coating produced from a 10:1 molar ratio of uncomplexed LN and FN stimulated locomotion ~90% of that over pure

FN Promotes the Formation of Vinculin-containing Focal Contacts and Organizes the Actin Cytoskeleton; LN Does Not

A reorganization of the cytoskeleton as cells go from stationary to motile states has been described in several systems. We therefore examined how components of the cytoskeleton redistributed with time following MM14dy attachment to LN or FN (Fig. 6). Within 6 h of attachment to FN, many cells had begun to co-opt vinculin into focal contacts, displayed organized actin stress fibers and exhibited α-actinin striations along the fibers (Fig. 6, *a–c*). In marked contrast, and as one would predict for motile cells, on LN cells seldom had vinculin-positive focal contacts, showed diffuse, weblike actin and only infrequently organized α-actinin into periodicities (Fig. 6, *d–f*). At higher coating concentrations of LN, the numbers of focal contacts and stress fibers increased (not

Table I. Pooled Data from Video Locomotion Assays

Substrate type	Average speed (μm h^{-1})	Average velocity (μm h^{-1})	Cells analyzed
LN	49 ± 6.6	20 ± 6.9 (n = 6)	92
E8 subfragment	36 ± 5.4	16 ± 5.1 (n = 5)	69
FN	14 ± 3.3	4.7 ± 1.5 (n = 5)	89
LN-nidogen	105 ± 3.8	38 ± 4.9 (n = 3)	57

MM14dy myoblasts locomoting over substrates coated for 1 h with 10 μg ml^{-1} solutions of LN, its subfragment E8, FN, or the LN-nidogen complex were analyzed as described in the Materials and Methods section, and the data from n independent experiments representing a total of >50 cells on each substrate was pooled. The standard deviations from each pool are shown. The individual experiments summarized here are plotted in Fig. 3 and are described in detail in the Materials and Methods section. *Speed*, (the length of the cell path)/(the time taken to travel it); *velocity*, (the distance of the cell from its starting point)/(the time taken to get there). This value is more exactly "the magnitude of the mean cell velocity."

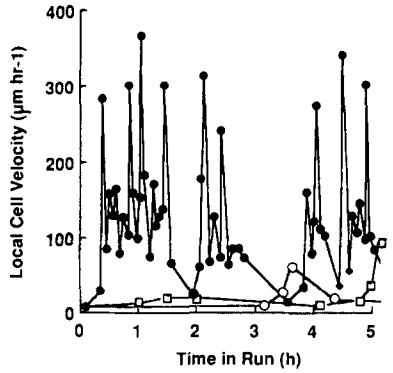


Figure 4. Myoblast locomotion over laminin is highly irregular. The local velocity of myoblasts over LN (*solid circles*) and FN (*open circles* and *squares*) was calculated for individual 5-μM steps along cell paths. Three representative cells are shown.

LN. However, when a large excess of FN was used mixed with LN-nidogen (10:1 molar ratio), the locomotion was essentially identical to that on the pure FN substrate. Thus, the negative effects of FN on locomotion appear to predominate as a signal over the positive effects of LN.

LN and FN Affect MM14dy Locomotion only as Substrate Coatings and Dominate over Soluble Components

It is likely that LN and FN bind to cells over specific cell surface receptors. We therefore attempted to modify the cell behavior on one substrate molecule by simultaneously providing the other molecule in soluble form, reasoning that it should occupy vacant receptors and provide a signal, positive for LN, negative for FN.

When we supplied exogenous LN to the medium of cells moving over FN, there was no stimulation of migration (Table III). Removal of endogenous FN from the culture medium also had no effect on cell locomotion over LN (not shown). These data are in marked contrast to the effect of substrate-adsorbed FN on locomotion over LN substrates (above). Thus, either the configuration of LN and FN molecules on the substrate is critical to their effects on cell locomotion, or the restraining of the cell surface ECM binding sites induces the motile phenotype.

When cells on LN were transferred into serum free medium (after being allowed to locomote in normal medium for 12 h) they continued to locomote for ~24 h before assum-

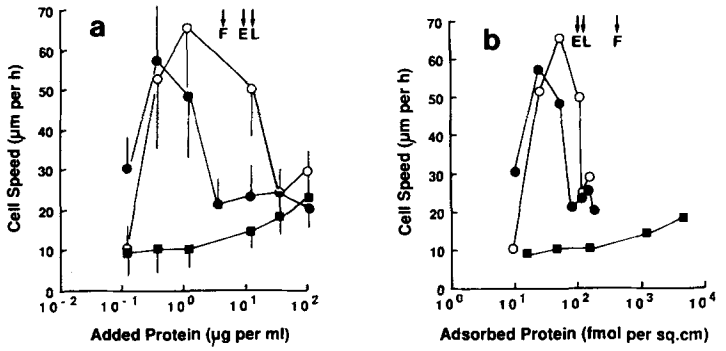
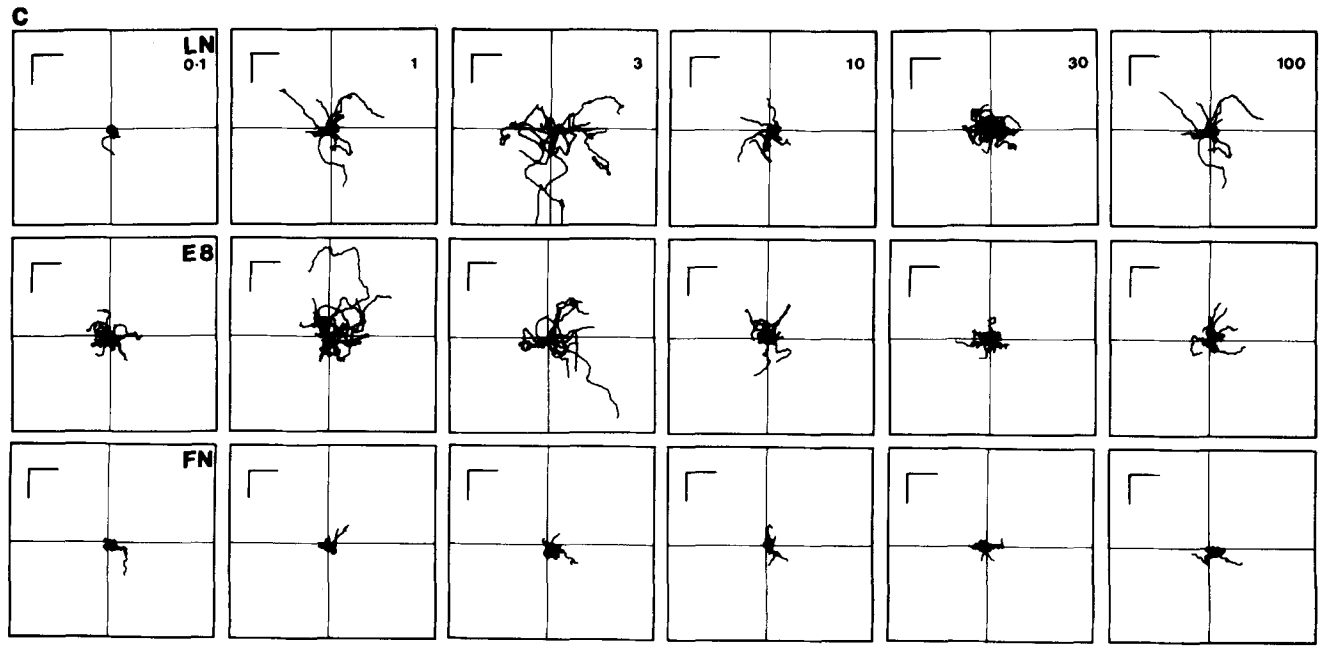


Figure 5. The locomotory response of myoblasts is highly dependent on the concentration of LN and E8 on the substrate but independent of the FN concentration. The locomotory response of myoblasts to substrates coated with increasing concentrations of LN (*open circles*), E8 subfragments (*solid circles*), or FN (*solid squares*) was measured by analytical video microscopy as described in detail in Materials and Methods. (*a*) the normalized data is plotted using the concentration of protein as abscissa. (*b*) same data but recalculated with the protein bound to the substrate as abscissa. (*c*) Normalized wind-rose plots through the concentration curves. Numbers represent coating concentration (μg ml^{-1}). Error bars, 1 SD. The arrowheads *F*, *L*, and *E*, show the coating concentrations where cell attachment to FN, LN, and E8, respectively, has reached a maximum (Goodman et al., 1987; Öcalan et al., 1988; and our unpublished observations).



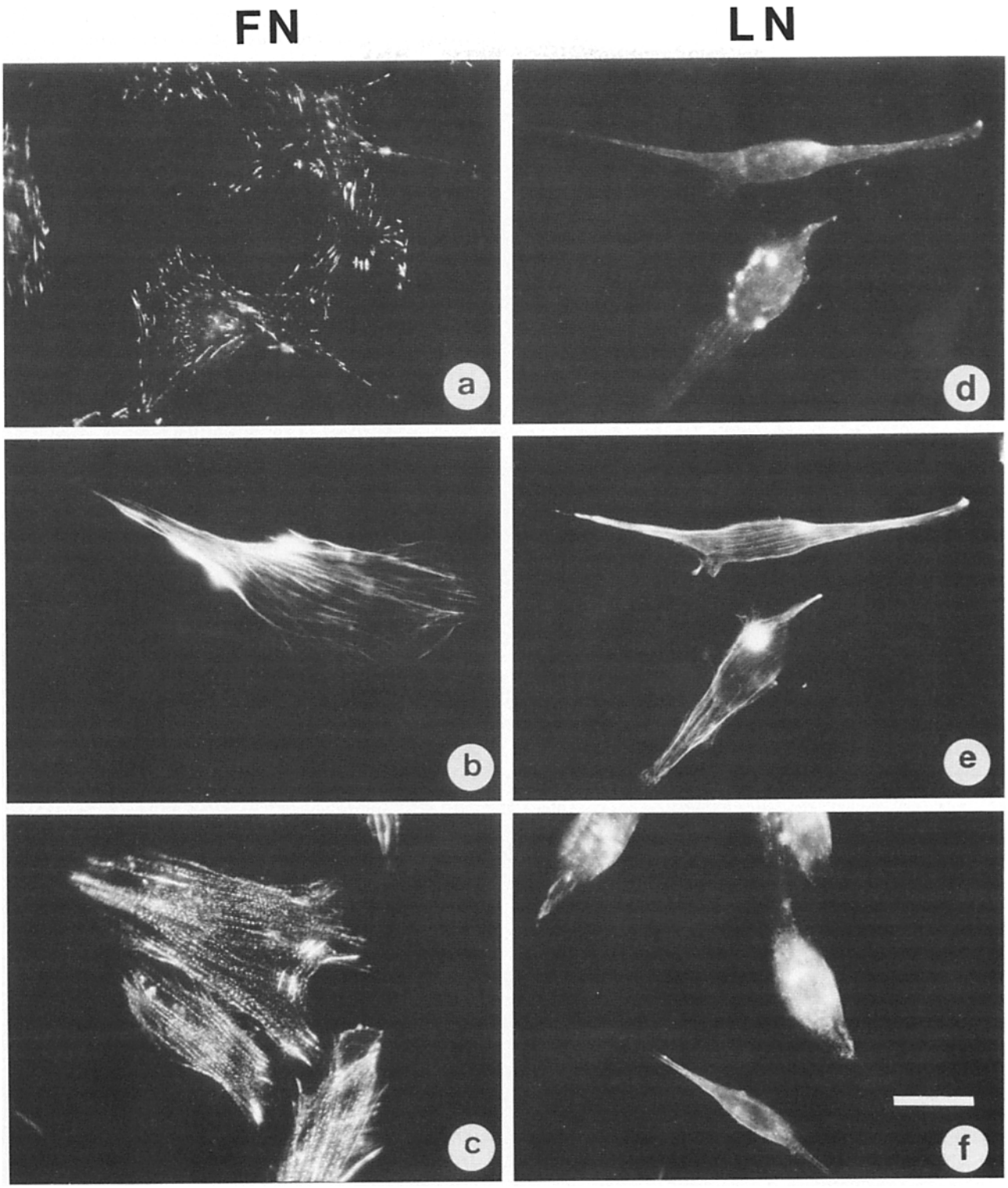


Figure 6. Specific reorganization of the cytoskeleton accompanies myoblast interactions with LN E8 and FN. Myoblasts 6 h after attachment to FN (*a*, *b*, and *c*) or LN (*d*, *e*, and *f*) were stained using indirect immunofluorescence techniques for cytoskeletal components vinculin (*a* and *d*), actin (*b* and *e*) or α actinin (*c* and *f*) as described in detail in Materials and Methods.

ing an extreme bipolar extended and stationary form (not shown). No difference in motion in serum free medium compared to serum-plus conditions was observed on FN-coated substrates (Table III). Thus, serum factors neither blocked motion over FN nor stimulated it over LN (in the short term).

Cell migration in vivo can be perturbed by modifying the interaction between cells and the LN-heparan sulphate proteoglycan complex (Bronner-Fraser and Lallier, 1988). We therefore tested whether the heparan binding sites in LN might affect locomotion by stripping LN substrates of associated heparan sulphate with heparinase (Table IV), and by attempting to saturate heparan binding on LN with heparin.

Table II. Effect of Mixed LN/FN Substrates on MM14dy Locomotion

Substrate type	Average speed ($\mu m\ h^{-1}$)	Average velocity ($\mu m\ h^{-1}$)	Cells analyzed
LN 10: FN 1*	45 ± 26	19 ± 26 (n = 1)§	18
FN 10: LN-nido 1‡	13 ± 12	9 ± 9 (n = 1)	13
FN 1: LN-nido 1	63 ± 16	21 ± 15 (n = 1)	17
LN‖	49	20	
FN‖	14	4.7	
LN-nidogen‖	105	38	

LN and FN were mixed in various molar ratios before adsorption onto the substrate and video-microscopic analyses as described in detail in the Materials and Methods.

* The numbers give the molar ratio of the molecules mixtures used at a final concentration of 10 $\mu g\ ml^{-1}$ for substrate coating.

‡ LN-nido = intact LN nidogen complex.

§ Where n = 1 or 2, the standard deviation is derived by considering the speeds and velocities for the individual cells in the culture.

‖ These values are abbreviated from Table I to aid comparison; complete data is in Table I.

Heparinase produced an ~30% reduction in locomotion over controls, while addition of heparin had no effect (not shown).

Laminin Analogues and Postulated Receptor Cofactors Do not Affect MM14dy Locomotion over LN

To identify which cell surface receptors were being used by locomoting myoblasts, we tested reagents believed to interact with LN receptors. A pentapeptide sequence from the B1 chain of murine LN, YIGSR, has been reported to mimic a cell binding site in LN (Graf et al., 1987). We therefore added high concentrations of YIGSR to MM14dy locomoting over LN-nidogen. It had no effect.

A cell surface 5'-nucleotidase activity can be activated after LN-cell interactions (Risse et al., 1987). This enzyme may therefore lie on a signal pathway between ECM and the cellular response. When we blocked the nucleotidase with AMP-CP, the locomotion of cells over LN was slightly stimulated. It slightly inhibited their locomotion over FN (Table IV).

Discussion

Cell locomotion is a complex phenomenon. Precise structural changes are initiated within the cell but the mechanisms by which these changes are effected and directed are largely unknown (for discussion, see Lackie, 1986; Bretscher, 1988). We used video microscopy to analyze in detail the locomotion of MM14dy murine muscle satellite cells. Our major findings are that (*a*) MM14dy move much more rapidly over LN than over FN; (*b*) the locomotion promoting activity of LN resides in the E8 subfragment; (*c*) the locomotion stimulation is distinct from pure adhesive effects; (*d*) only the interaction of cells with molecules on the substrate, not in solution, triggers the definitive motile response and finally; (*e*) the motile responses are accompanied by characteristic substrate-dependent redistributions of the cytoskeleton.

LN strongly stimulates myoblast locomotion while FN does not. Our results are in apparent contrast to studies on primary avian skeletal muscle cells that move over FN (Turner et al., 1983). However, these assays were long and nonquantitative, and no comparison with LN was made; thus, comparisons with our data are not straightforward. Furthermore, the LN and FN receptors of avians appear different from those of mammals. In avians, cell attachment to LN and FN involves receptors recognized and perturbed by the anti-CSAT antibody and dependent on the peptide sequence RGDS- (Horwitz et al., 1985; Ruoslathi, 1988). By contrast, in murine and human cells, several RGDS-independent receptors bind LN, while the FN receptor is RGDS dependent (Goodman et al., 1987; Dillner et al., 1988). Early work with neural crest cells showing that FN was responsible for cell locomotion (Newgreen and Thiery, 1980) seems also to have been an oversimplification of the situation in vitro (Newgreen, 1984; Goodman and Newgreen, 1985) and in vivo (Bronner-Fraser and Lallier, 1988). For myoblasts in vivo, the question is still open whether FN or LN are used as substrates for migration during development or repair. However, their importance is suggested by the finding that embryonic implants of anti-CSAT-producing hybridoma cells lead to perturbed migration of avian myoblasts (Jaffredo et al., 1988).

Murine myoblasts attach to LN, E8, and FN, but respond by moving differently. The data were obtained using two very different measuring techniques, a qualitative "stripe" assay, using high cell densities, and quantitative video-microscopy where low densities were used. The major conclusions to be drawn from both assays are identical. On LN and E8, myoblasts move rapidly and on FN very slowly. We show here that the necessary locomotion stimulating activity of LN alone is present in the E8 subfragment. However, the LN-nidogen complex produces a twofold enhancement of their locomotion rates over LN alone. There are three simple explanations for this; first, the complex is isolated in physiological as opposed to high salt concentrations and may be less denatured than LN. Second, the nidogen-LN interaction may induce structural changes in one or other of the molecules that creates a locomotion-stimulatory site. Alternatively, nidogen or LN-nidogen-associated molecules may also stimulate cell locomotion. These alternatives require further investigation.

Table III. Effect of Altering the Concentration of Soluble Matrix and Serum Components on MM14dy Locomotion over LN and FN

Substrate type	Average speed ($\mu m\ h^{-1}$)	Average velocity ($\mu m\ h^{-1}$)	Cells analyzed
FN*	13 ± 8.9	4 ± 3.5 (n = 1)	12
FN + soluble LN*	15 ± 15	3 ± 3.8 (n = 1)	10
FN	19 ± 14	6 ± 7.6 (n = 1)	16
FN serum free	22 ± 7.5	11 ± 5.3 (n = 1)	12
LN	52 ± 18	9 ± 7.1 (n = 1)	22
LN serum free	55 ± 25	22 ± 19 (n = 2)	16
LN‡	49	20	
FN‡	14	4.7	

Cells were allowed to locomote on in control conditions for 12 h before either addition of LN (end concentration 100 $\mu g\ ml^{-1}$), or washing out the serum with serum free medium (+0.5% BSA), and continuing the run. For further details, see the legends to Tables I and II and the Materials and Methods section.

* In each case, the values for cultures before and after change of conditions is given.

‡ These values are abbreviated from table I to aid comparison.

Table IV. Effect of Potential Perturbers of LN Receptor–Substrate Interactions

Substrate type	Average speed ($\mu m\ h^{-1}$)	Average velocity ($\mu m\ h^{-1}$)		Cells analyzed
FN + AMP-CP	9 ± 7.1	4 ± 4	(n = 1)	19
LN + AMP-CP	71 ± 35	30 ± 27	(n = 2)	38
LN-nido‡ + YIGSR	103 ± 49	32 ± 28	(n = 1)	17
LN-nido + heparinase	72 ± 33	20 ± 14	(n = 1)	19
FN*	14	4.7		
LN-nido*	105	38		
LN*	49	20		

MM14dy were analyzed by video microscopy while locomoting in the presence of AMP-CP, YIGSR, or over heparinase-treated substrates as described in detail in Materials and Methods.
* These values are abbreviated from Table I to aid comparison.
‡ LN-nido, intact LN nidogen complex.

The morphology of myoblasts locomoting over LN and E8 is very similar to that of neural crest cells moving through a collagen matrix (Tucker and Erickson, 1984), and differs from fibroblast locomotion. E8 is the fragment responsible for stimulating neurite outgrowth on LN substrates (Edgar et al., 1984). Since the morphology of the lamellipodia of migrating MM14dy strongly resembles outgrowths of neurites, it is tempting to suggest that similar molecular processes are involved. Differences in the quantities of E8 required for neurite outgrowth (~10 ng ml^{-1} coatings) over those needed for myoblast locomotion (~500ng ml^{-1}) are most likely the result of the adsorption properties of the polyornithine used to permit neurone attachment (Dr. D. Edgar, personal communication). Not only does E8 bear regions necessary for stimulating locomotion, but, interestingly, it does so at the same coating density as LN. This agrees with recently published work indicating that the binding of many cells, including MM14dy, to LN operates over cell surface E8 binding sites apparently identical (similar affinity and number present) to those for LN (Aumailley et al., 1987; Goodman et al., 1989*a*). Thus, it is comforting that the same concentration of triggering stimulus provokes the same cellular responses.

The locomotory stimulus varies dramatically with the density of substrate-bound E8 and LN but over a wide concentration range does not for FN. The question arises of whether this effect is because of simple variations in adhesivity; too high or too low adhesion rendering the cells immobile, intermediate levels permiting locomotion. We coated with concentrations of both LN and FN ranging from those where cells were rounded to those that produced extremely flattened and well spread cells. Previous detailed cell attachment studies (Goodman et al., 1987; Öcalan et al., 1988) and everyday observation (rounded cells come off the substrate when the plates are tapped or washed to apply shearing forces, while flattened cells are highly resistant to such forces) suggest that the interaction between cells and LN or FN increases with increasing concentration of coating; cells adhere more strongly at high concentrations. Furthermore, the adhesion of MM14dy to LN and FN shows similar kinetic and concentration dependency for attachment and spreading (Öcalan et al., 1988). Although the mechanisms involved are complex, such data in effect measure the affinity of the cells for the substrates and suggest that the gross strength of adhesion to similar concentrations of LN and FN is similar. Thus, the contrasting response of MM14dy to LN and E8, and to FN has a basis other than in variation of adhesion alone. The relatively sharp response of the myoblasts to specific concentrations of LN is consistent with the idea that following binding of sufficient matrix to cell surface binding sites, a "triggering" level of a specific signal is generated within the cytoplasm. Although myoblasts must attach to locomote, our data clearly show that adhesion alone is not sufficient to induce locomotion. Several candidate LN receptors have been described (for review, see Timpl and Dziadek, 1987; Risse et al., 1987). Possibly one cell surface binding site initiates primary cell attachment, while subsequently another one triggers locomotion, as is implied for FN (McCarthy et al., 1986).

Cells must adhere before they can locomote, and it has been suggested that they may navigate by detecting and using adhesive differences in their environment ("haptotaxis"; Carter, 1965). LN and E8 stimulate MM14dy locomotion at low concentrations while at high concentrations they stop MM14dy moving, a pattern predicted for molecules that act haptotactically. Our data are in general agreement with data on LN-induced haptotaxis using B16 melanoma cells (McCarthy and Furcht, 1984) where a peaked response to LN was also seen. McKenna and Raper (1988) recently reported a similar dose response for induction of neurite outgrowth to the one we report here for MM14dy locomotion, but, at LN coatings of up to ~700 fM cm^{-2}, they saw no decrease in locomotory response. The lack of myoblasts' kinetic response to high concentrations of LN and E8 may be because of their strong attachment or could be related to high receptor occupancy (cf., chemotactic receptors; Tranquillo et al., 1988).

One of the rapid responses to variations in the ECM involves changes in the cytoskeleton, a structure implicated in both motile phenomena and in specific gene activation. In particular, in locomotory cells stress fibres, vinculin-positive focal contacts and periodically arranged α-actinin are characteristically sparse (Abercrombie et al., 1971; Lazarides and Burridge, 1975; Couchman and Rees, 1979; Burridge and Feramisco, 1980; Couchman et al., 1982; Duband et al., 1988). This type of organization is visible in MM14dy on LN. On FN, actin and α actinin rapidly organize into stress fibers and vinculin into focal contacts, characteristics of immobile cells. The results thus corroborate and extend data from our video-microscopy and "stripe" assays and indicate that matrix components induce specific and drastic changes in the myoblast cytoskeleton. Matrix receptors can colocalize with vinculin and talin at focal contacts (Damsky et al., 1985; Horwitz et al., 1986; Burridge et al., 1987), and transferring cells onto different substrates can leave focal contacts intact but disperse the previously relevant matrix receptors (Duband et al., 1988). Our data further supports the idea that the stability of focal adhesions and the recruitment of their various components (*a*) is critically dependent on the motile state of the cell; and (*b*) can be controlled by specific components of the ECM. Thus cell behavior on FN may be a result of its ability to trigger cytoskeletal stabilization.

LN only stimulates locomotion as substrate; in solution it neither stimulates motion over FN, nor does soluble FN inhibit locomotion on LN. This may be because only im-

mobilization and clustering of matrix binding sites at the substrate can give cells the necessary signals, or because the effective concentration of matrix molecule on the substrate is higher than the concentration in solution.

A theoretical basis for the ability of substrates to influence myoblasts locomotion can be found in studies on neutrophils and fibroblasts (Dunn and Brown, 1987). These suggest that the current state of the cell (here we would include the substrate molecules it is detecting) alone determines what it will do next; cells have no long-term locomotory memory (Levinstone et al., 1983; Dunn and Brown, 1987). Thus, substrates can rapidly influence cell locomotory behavior; i.e., myoblasts need not remain stationary because once they were stationary, nor move because once they moved.

Interaction of myoblasts with different matrix components triggers different motile behavior. Our data supports a model where different cellular responses to LN and FN play a role (*a*) in maintaining myoblasts in a preterminally differentiated state; (*b*) in supporting their migration through the embryo; (*c*) in accelerating the myoblast differentiation program; and (*d*) in trapping myoblasts at their final destination. It is clear that myoblasts will continue to be an extremely interesting and fruitful system for analyzing matrix dependent signaling processes.

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