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Controlled induction of focal adhesion disassembly and migration in primary fibroblasts

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SUMMARY

Fibroblast migration is an integral component of biological processes such as wound healing and embryogenesis. Previous experiments examining fibroblast locomotion from tissue explants have shown that migrating fibroblasts lack, or contain only transient, focal adhesions (focal contacts). Focal adhesions are specialized regions of tight cell-matrix interaction, assembled by a complex process of transmembrane signalling. Although the explant model has been used for studying several aspects of fibroblast locomotion, it is limited by the lack of control over migration, and only a small percentage of the cells actually locomoting. Therefore, we have developed an in vitro model for cultured fibroblast strains where the presence or absence of focal adhesions can be manipulated, and in the latter case 70% of these cells become locomotory.

The stimulus used to decrease the percentage of cells containing focal adhesions, and hence enhance locomotion, was newborn rat heart-conditioned medium (HCM). Addition of HCM to rat embryo fibroblasts induced both chemokinesis and chemotaxis. Cells disassembled focal adhesions on a variety of extracellular matrix substrates after approximately 6 h of stimulation with HCM; conversely, removal of HCM promoted reformation of focal adhesions within 12-24 h. HCM-stimulated fibroblasts which lacked focal adhesions concomitantly lacked F-actin stress fibers and focal concentrations of vinculin and talin. Therefore, fibroblast migration can be readily controlled in an on-off manner through conditioned medium, which influences the absence or presence of focal adhesions.

Key words: migration, focal adhesions, fibroblast, chemokinesis, chemotaxis

INTRODUCTION

Focal adhesions are discrete transmembrane assemblies, in which complexes of cytoskeletal and membrane components are tightly associated with the underlying extracellular matrix, and can occur in fibroblasts, platelets, smooth muscle, endothelial and epithelial cells (Burridge et al., 1988; Woods and Couchman, 1988). Numerous proteins are enriched in focal adhesions (for reviews, see Burridge et al., 1988; Woods and Couchman, 1988) and immunolocalization of some, for example vinculin and talin, can be used to identify these structures. In addition, a useful technique for visualizing focal adhesions, particularly in living cells, is interference reflection microscopy (IRM) (Izzard and Lochner, 1976; Couchman and Rees, 1979b). Highly locomotory fibroblasts emerging from embryo heart explants generally lack, or contain only transient, focal structures and associated F-actin microfilament bundles (Small et al., 1978; Badley et al., 1980; Herman et al., 1981; Heath and Holifield, 1991; Theriot and Mitchison, 1991). For adhesion to the substrate during locomotion, a much broader contact called a close contact is usually employed (Izzard and Lochner, 1976; Couchman and Rees, 1979b). These cells could migrate at rates up to 80 μm/h during an initial 48-72 h period, after which they spontaneously and irreversibly converted to a stationary phenotype with the development of many focal adhesions (Couchman and Rees, 1979b).

Fibroblasts migrate during embryogenesis and wound healing, and in the transformed state. They can migrate both chemotactically in response to a gradient of stimulant, and chemokinetically, by random cell migration, in response to a non-directional, uniform concentration of factor(s). There are several known chemotactic factors for fibroblasts, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), migration stimulating factor (MSF), transforming growth factor β (TGF-β), and tumor necrosis factor α (TNF-α) (Grotendorst, 1988; Schor et al., 1988a,b; Adelmann-Grill et al., 1990; Postlethwaite and Seyer, 1990). Fibroblasts also migrate chemotactically towards some matrix molecules or their fragments, including fibronectin, the 'cell-binding' domain of fibronectin (Ruoslahti, 1988; Hynes, 1990), the Arg-Gly-Asp-containing hexapeptide GRGDSP, types I-III collagen, tropoelastin and elastin peptides (Senior et al., 1982, 1984; Albini et al., 1983, 1985; Long et al., 1987, 1988; Hynes, 1990). These

studies have been extremely useful in determining which molecules stimulate fibroblast migration, but were not designed primarily to investigate the mechanics of fibroblast migration.

Morphological details of primary fibroblast migration have been previously examined using phase contrast, electron and interference reflection (IRM) microscopy, often using fibroblasts migrating from embryonic heart explants as the source of locomoting cells (Abercrombie et al., 1971, 1980; Izzard and Lochner, 1976, 1980; Heath and Dunn, 1978; Couchman and Rees, 1979a,b). These cells were found to be polarized, with a well defined leading lamella and a trailing edge (Chen, 1979, 1981; Izzard and Lochner, 1980; Heath, 1983). The leading lamella is thin and generally devoid of cellular organelles, but does contain elevated concentrations of F-actin and talin (Small and Langanger, 1981; Couchman and Rees, 1982; DePasquale and Izzard, 1991). The use of IRM to study migratory fibroblasts also revealed that the leading lamella lacked focal adhesions, although transient focal contacts were sometimes found just proximal to this region (Izzard and Lochner, 1976; Couchman and Rees, 1979b).

Our interest has been to develop a system whereby controlling the presence of focal adhesions in fibroblasts also alters the migratory state of these cells. This is difficult with chemotaxis systems and other models such as tissue explants or 'scratched' monolayer cultures, because in such systems there is no control over the migration process, there is a lack of uniformity, and the percentage of cells undergoing migration can be relatively small. Since routinely cultured fibroblasts contain many focal adhesions, and are generally 'stationary', the main focus of such experiments was the determination of which factors, or combination of factors, would stimulate fibroblasts to disassemble their focal adhesions and undergo chemokinesis. We have developed a novel migration model for cultured primary fibroblasts in which the absence or presence of focal adhesions in the majority of these cells can be manipulated by the addition or removal of heart-conditioned medium (HCM). We show here that rat embryo fibroblasts (REF) respond to HCM in a dose-dependent manner by disassembling focal adhesions, decreasing mitotic rate and undergoing both chemotactic and chemokinetic locomotion. Furthermore, this process can be manipulated in an on-off manner and is associated with profound changes in cytoskeletal and cell-substrate adhesion characteristics.

MATERIALS AND METHODS

Materials

General chemicals, bovine serum albumin (BSA), EM-grade glutaraldehyde, and hematoxylin were purchased from Sigma Chemical Company (St. Louis, MO). Paraformaldehyde, permount, isopropanol, Nalgene filters (0.20 μm pore size), coverslips and tissue culture plasticware were from Fisher Scientific (Atlanta, GA). Tissue culture media were from Mediatech (Washington, DC), fetal bovine serum (FBS) was from HyClone Laboratories (Logan, Utah) and glutamine, penicillin/streptomycin, kanamycin and Linbro 24-well tissue culture plates were from Flow Laboratories (Costa Mesa, CA).

The modified Boyden chamber was obtained from Guy Duremberg (Pasadena, CA), PVP (polyvinylpyrrolidone)-free polycarbonate membranes of 8 μm pore size and 13 mm diameter, were from Nucleopore (Pleasanton, CA) and cellulose nitrate membranes of 0.45 μm pore size and 13 mm diameter, were purchased from Millipore Corporation (Bedford, MA).

Monoclonal mouse anti-vinculin IgG was obtained from Sigma, and polyclonal rabbit anti-talin antiserum was a kind gift from Dr K. Burridge, (University of North Carolina at Chapel Hill). Affinity purified FITC-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG and TRITC-conjugated $F(ab')_2$ fragment of goat anti-rabbit IgG were from Organon Teknika Corporation, (West Chester, PA). TRITC-conjugated phalloidin was obtained from Molecular Probes Inc. (Eugene, OR).

Human platelet-derived growth factor (PDGF) and human acidic fibroblast growth factor (aFGF) were from Intergen Corporation (Purchase, NY). Epidermal growth factor (EGF) was purified from mouse submaxillary gland and was a kind gift from Dr J. E. Kudlow (University of Alabama at Birmingham). Human transforming growth factor β_1 (TGF-β_1) was from Genzyme Corp. (Cambridge, MA) and human basic fibroblast growth factor (bFGF) was from R & D Systems (Minneapolis, MN).

Type I collagen (Vitrogen) was from Collagen Corporation (Palo Alto, CA); laminin, fibronectin and the 105 kDa 'cell-binding' domain of fibronectin were generously provided by Dr D. R. Abrahamson and Dr M. R. Austria (University of Alabama at Birmingham).

Cell culture

Rat embryo fibroblasts (REF) (Woods et al., 1988; Badley et al., 1981) and human embryo fibroblasts (HEF) (a gift from Dr K. Hedman, Department of Virology, University of Helsinki, Finland) were grown in αMEM containing 10% or 5% FBS, respectively. Human gingival fibroblasts (HGF), Balb/c-3T3 fibroblasts and RAT-1 (F-2408) cells were obtained from Dr Charles Prince (University of Alabama at Birmingham), ATCC (American Type Culture Collection) and Dr J. Schwarzbauer (Princeton University, NJ) respectively, and were grown in DMEM containing 10% FBS. Trypsin/EDTA was used to passage the cells every 4-5 d. Primary cell culture strains, including REF and HGF, were used in experiments before the 30th passage and HEF were used before the 5th passage. For chemokinesis, migration and chemotaxis assays, REF were grown for 4 d following a 1:5 split ratio. All cell cultures and experiments were performed at 37°C in 10% CO_2.

Conditioned media from HGF, RAT-1 and Balb/c-3T3 cells were obtained by removing the spent medium after 4-5 d cell growth from a 1:10 split ratio through to subconfluency. HEF-conditioned medium (HEF-CM) was obtained from cells that had grown for 6-7 d from a 1:2 split ratio through to subconfluency. Since HCM contained antibiotics, control REF-conditioned medium (REF-CM) was prepared by growing cells for 3 d, then culturing the cells for 48 h in αMEM containing 10% FBS, 50 i.u./ml penicillin, 50 μg/ml streptomycin and 50 μg/ml kanamycin. This allowed us to discount any effects of antibiotics on locomotion. All conditioned media were centrifuged in a Beckman TJ-6 centrifuge at 1000 r.p.m. for 12 min, filtered through a Nalgene 0.20 μm cellulose nitrate membrane and then stored at −20°C. All cultured cells were routinely screened for mycoplasma and found to be uncontaminated.

Heart-conditioned medium (HCM) collection

Hearts were dissected from 12-48 h old Sprague-Dawley rats, and the ventricles finely minced with a scalpel blade before culturing in αMEM with antibiotics (as above), 4 hearts/25 cm^2 tissue culture flask. The first serum-free HCM collection was removed after

24 h and replaced with αMEM containing 10% FBS and antibiotics. From this point on, collections of HCM each containing FBS, were made every 48 h, for a total of 11 consecutive collections. HCM was removed, centrifuged and filtered, as above, and stored at −20°C.

Chemokinesis assay

REF were trypsinized and seeded at 4×10^4 cells/ml for incubation times of ≤12 h, or at 2×10^4 cells/ml for incubation times >12 h. Glass coverslips (12 mm diameter) were coated with FBS by incubation for a minimum of 1 h with αMEM containing 10% FBS in 24-well plates, before addition of 0.5 ml of the appropriate cell suspension. Unless otherwise indicated, the cells were allowed to attach and spread on the coverslips for 1 h before replacement of the supernatant with HCM or one of two control media (REF-CM or αMEM containing 5% FBS and antibiotics). Cells were incubated in HCM or control media for the indicated time points, then fixed in 3.0% glutaraldehyde in phosphate-buffered saline (PBS) for 30 min at 37°C. Coverslips were washed 3×15 min in PBS containing 1.0 mM Ca^{2+} and 1.2 mM Mg^{2+} and mounted onto glass slides in the same buffer. The cells were examined by IRM and the percentage containing focal adhesions was quantitated by counting a minimum of 100 cells per coverslip. Each experimental condition was performed in duplicate and all experiments were repeated using different HCM collections.

Fluorescence staining

For indirect immunofluorescence microscopy or staining of F-actin, REF cells were seeded on glass coverslips and treated for 12-16 h with HCM or control media, as described in the chemokinesis assay. For immunofluorescence, cells were fixed for 20 min in 3.5% freshly hydrolyzed paraformaldehyde in PBS at room temperature or in paraformaldehyde with 0.1% Tween-20, at 37°C for 5 min. For staining of F-actin, cells were fixed in 3% glutaraldehyde in PBS. Permeabilization, staining and mounting procedures were as previously described (Woods et al., 1986; Woods and Couchman, 1992), where a 1:25 or 1:50 dilution of mouse anti-vinculin, a 1:100 dilution of rabbit anti-talin, a 1:40 dilution of secondary antibody, FITC-conjugated goat anti-mouse IgG or TRITC-conjugated goat anti-rabbit IgG, or 2 U/ml (0.07 μM) of rhodamine-phalloidin in PBS, were used.

Microscopy

Cells were routinely examined on a Nikon Optiphot microscope equipped with epifluorescence, phase contrast and interference reflection microscopy optics (Woods and Couchman, 1992). All photographs were taken using Ilford HP5 film.

Cell migration assay (video microscopy)

REF were trypsinized and seeded at a concentration of 2×10^4 cells/coverslip on FBS-coated 25 mm glass coverslips for 1 h at 37°C. The supernatant was then replaced with HCM or REF-CM and cells were incubated for 12-16 h. Coverslips were then mounted in a thermally controlled stainless steel chamber and placed on a Zeiss Axiovert 35 microscope. Using a digital imaging system, migration rates were determined by recording the nuclear displacement of cells in three different low power fields (×10 or ×20 objective) every 6-7 min over a 6-7 h period. This system consisted of a Dage MTI model 72 CCD camera interfaced with a DATA Cube imaging subsystem, a Sun 3/260 work station, a Sony super fine pitch monitor and a Mitsubishi color video copy processor, with imaging software by Inovision.

Growth factor assay

Cells were prepared and treated as described in the chemokinesis assay, in which REF were allow to attach and spread for 1 h before a 12-16 h incubation with various growth factors diluted in αMEM. FBS was added to each sample to a final concentration of 5%, in order to best represent the conditions in HCM and the control media. All experimental coverslips were prepared in quadruplicate.

Chemotaxis assay

Chemotaxis was measured using modified Boyden chambers, as previously described (Senior et al., 1984; Long et al., 1988). Briefly, 0.45 μm Millipore membranes were serum-coated for 1 h in αMEM containing 10% FBS and antibiotics, then washed 3×5 min in PBS. The chamber was assembled by adding 0.24 ml of HCM or control media to the bottom compartment, followed by the 0.45 μm membrane and then the 8 μm membrane. Next, 0.37 ml of REF suspension (4×10^4 cells/ml), in αMEM containing 5% FBS and antibiotics or in the indicated conditioned medium, was added to the upper compartment. After incubation for 6 h, medium in the top chamber was removed and both membranes were fixed in 3.5% paraformaldehyde in PBS for 20 min. The membranes were placed in ethanol, stained in Harris' hematoxylin, dehydrated in a graded series of isopropanol, cleared in xylene and mounted in permount onto glass slides. The number of cells remaining on top of the 8 μm membrane and the number that migrated through the 8 μm membrane (including those on the bottom of the 8 μm membrane and those trapped on top of the 0.45 μm membrane), were counted in five high power fields (×320) using bright field optics. Data is shown as the percentage of the total cells counted that had migrated through the 8 μm membrane. All experimental parameters were tested in duplicate or triplicate and experiments were repeated using different HCM collections.

Chemokinesis on various extracellular matrix molecules

Cells were prepared and treated as described in the chemokinesis assay, with the exception of coverslip treatments. Glass coverslips were prepared by absorbing 10 μg of type I collagen (dissolved in 0.1% acetic acid) or 2 μg of fibronectin at 4°C, or FBS at 37°C, for 12-16 h. Other coated substrata were prepared by air-drying 10 μg laminin or 4 μg of the 105 kDa fragment of fibronectin onto the coverslips for 12-16 h. All coverslips were rinsed 3×5 min in PBS followed by treatment with 1% heat denatured BSA for 1 h, and 3×5 min washes in PBS.

RESULTS

Focal adhesion dispersion and cytoskeletal alterations induced by HCM

Previous results have shown that highly locomotory fibroblasts lacked, or contained only transient, focal contacts, the premise being that fibroblast migration is correlated with the lack or transience of these structures. To test whether HCM could affect the presence of focal adhesions and hence enhance migration, the IRM image and cytoskeletal components of REF cells treated with HCM or control media were monitored. Using TRITC-conjugated phalloidin to stain F-actin, cells treated for 12-16 h with HCM were compared with those treated with control supernatants. Results showed that cells treated with control media, either αMEM containing 5% FBS (Fig. 1A,B) or REF-CM (Fig. 1C,D), and that contained focal adhesions, had F-actin localized in large stress fibers. However, cells that were incubated with HCM and lacked focal adhesions, had F-

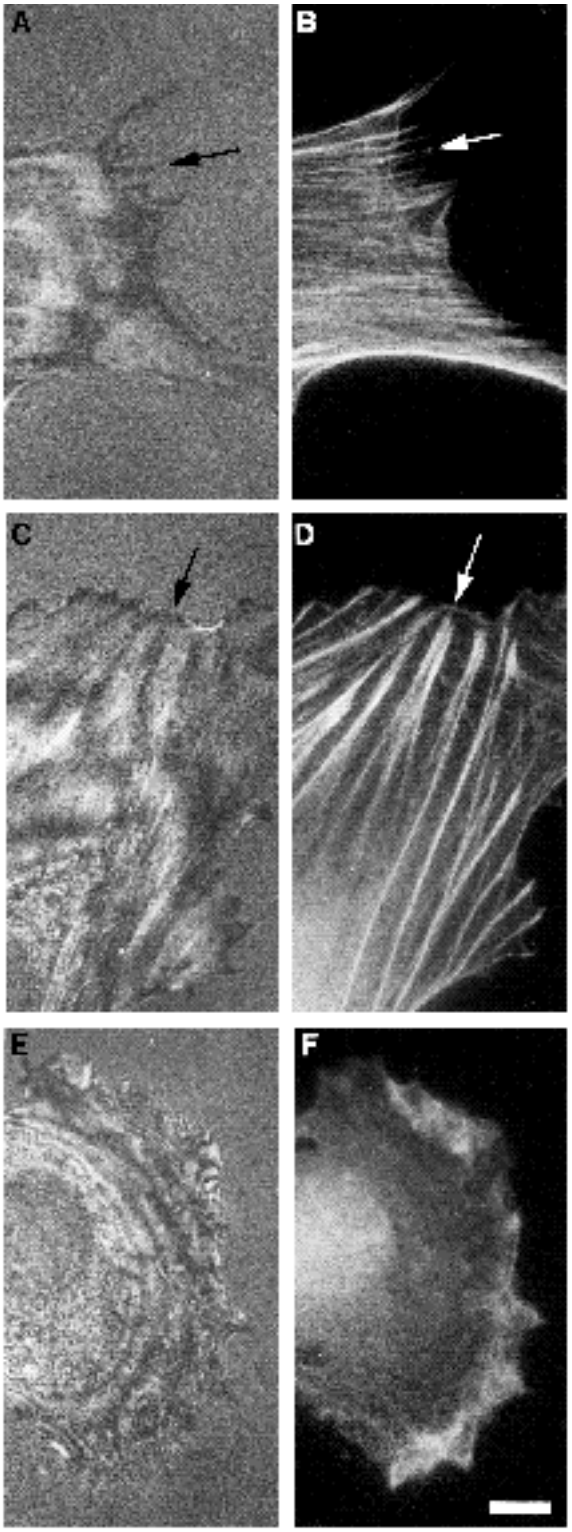

Fig. 1. HCM stimulates a loss of focal adhesions and associated F-actin stress fibers. REF cells were incubated for 12-16 h in αMEM with 5% FBS (A,B), REF-CM (C,D) or HCM (E,F). IRM images (A,C,E) and the corresponding F-actin localizations are shown (B,D,F). Arrows indicate where some of the large F-actin stress fibers terminated in focal adhesions in cells treated with αMEM with 5% FBS or REF-CM. However, most HCM-treated cells lacked F-actin stress fibers and focal adhesions but did contain a concentration of F-actin in the leading lamella. Bar, 5 μm

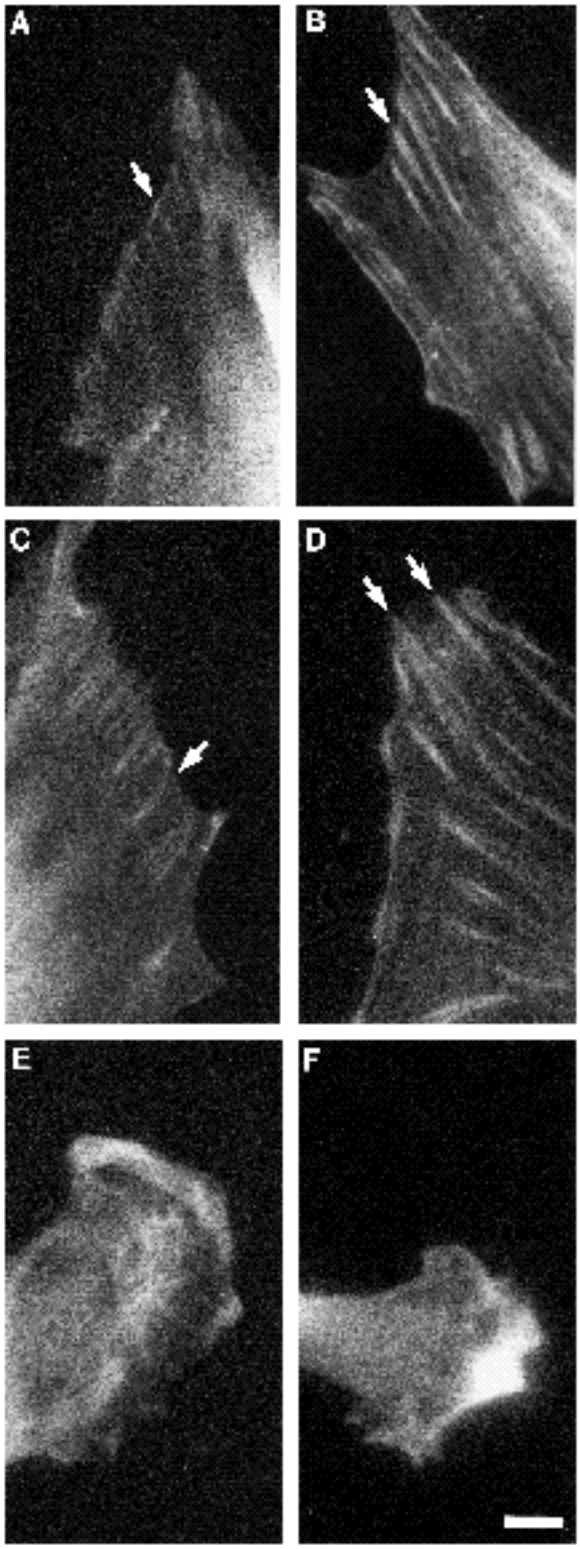

Fig. 2. Immunolocalization of vinculin and talin in cells treated with HCM or control medium. REF cells were treated with αMEM with 5% FBS (A,B), REF-CM (C,D) or HCM (E,F) for 12-16 h, fixed and stained for vinculin (A,C,E) or talin (B,D,F). Cells treated with control medium contained vinculin and talin in a punctate pattern representative of focal adhesion staining (arrows), whereas HCM-treated cells showed a diffuse immunolocalization for both vinculin and talin. Bar, 5 μm.

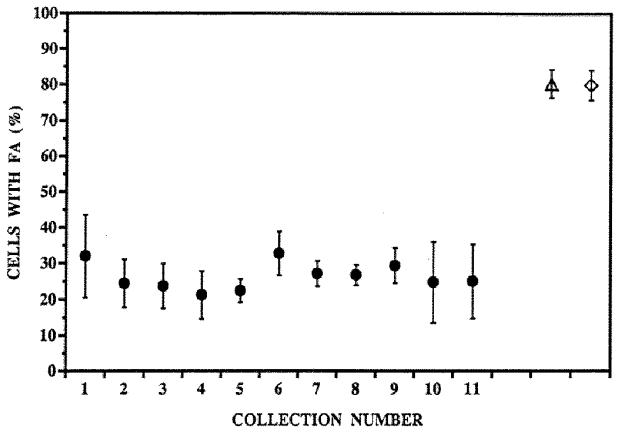

Fig. 3. The loss of focal adhesions upon HCM stimulation was quantitated using IRM. REF cells were incubated for 6 h in various collections of HCM (●), REF-CM (△) or αMEM with 5% FBS (◇). HCM collection numbers represent the chronological order in which supernatants were removed from the minced hearts, with no.1 being serum free and all others containing FBS. Data are represented by the percentage of cells with focal adhesions ± s.e.m. Each data point represents counts of 100 cells (minimum) on duplicate coverslips for each experimental condition; *n*=3-5 for HCM and *n*=3 for control media.

actin diffusely distributed throughout the cell with small concentrations in the leading lamella (Fig. 1E,F).

The distributions of vinculin and talin, which are cytoplasmic components of focal adhesions, were also examined by indirect immunofluorescence microscopy. In controls, treated with αMEM containing 5% FBS (Fig. 2A,B) or with REF-CM (Fig. 2C,D), both vinculin and talin were present in short linear streaks, consistent with their localization in focal adhesions. Conversely, HCM-treated cells (Fig. 2E,F) had vinculin and talin distributed diffusely, with an additional concentration of talin in the leading lamella. Therefore, in the majority of cells treated with HCM, there was a loss of focal adhesions as determined by both IRM and immunolocalization of cytoskeletal components.

Table 1. Video microscopy analysis of REF cells incubated with REF-CM or HCM

A

Supernatant	Migratory	Stationary	Mitosis	Fields of cells
REF-CM	38.8±8.6	31.9±15.9	35.4±19.8 (6.0±1.8)	*n*=3
HCM	76.1±11.1	8.4±6.4	20.0±11.8 (3.4±1.7)	*n*=9

B

Supernatant	Avg. rate	Avg. max	Avg. min	Cells observed
REF-CM	30.4±7.0	90.7±25.3	0±0	*n*=24
HCM	39.1±4.0*	140.2±14.1	1.5±4.2	*n*=27

The percentage of cells ± s.d. which were migratory, stationary or underwent mitosis during the 6-7 h experiment are shown in (A). The numbers in parentheses in the mitosis column represent the percentage of cells which underwent mitosis per h ± s.d. If a cell was stationary or migratory before undergoing mitosis it was scored twice, once as being stationary or migratory and then again as undergoing mitosis. The averages of the overall migration rate, and maximum and minimum migration rate were determined for individual cells stimulated with REF-CM or HCM (shown in B). The numbers represent rates in μm per h ± s.d. for REF-CM samples and ± s.e.m. for HCM samples. Data are from 3 fields of cells for REF-CM samples and from 9 fields of cells for HCM samples. *$P < 0.05$ for the average rate between REF-CM and HCM samples.

REF chemokinesis

The percentage of cells containing focal adhesions when incubated with HCM or control media was quantitated using IRM, as shown in Fig. 1A,C,E. Fig. 3 shows the striking difference between control and HCM-treated cells with respect to the number of cells which contain focal adhesions. When REF were incubated for 6 h in control media (either REF-CM or αMEM containing 5% FBS), approximately 80% of the cell population contained focal adhesions. However, when REF were incubated for the same time period in any of the HCM samples, only 21-32% of the cell population contained focal adhesions. The potency of this effect after dilution of HCM or REF-CM with αMEM containing 5% FBS was also examined (data not shown). The majority of REF exhibited a loss of focal adhesions up to a 1:20 dilution in HCM; however, much of this effect was lost at a 1:50 dilution. Conversely, the same dilutions with REF-CM yielded little decrease in the percentage of cells with focal adhesions (>68% cells having focal adhesions). HCM was never observed to stimulate a loss of focal adhesions in every cell, indicating the possibility that these fibroblasts comprise a heterogeneous population. Chemokinetic ability was based on the lack of focal adhesions in migratory cells and the presence of many of these structures in stationary cells.

To verify that this method of estimating chemokinesis was closely correlated with actual locomotory activity, a series of phase contrast video microscopy experiments were performed. These determined the percentage of REF, incubated with either HCM or REF-CM, which underwent locomotion and the rate at which the cells migrated. The majority of cells incubated with HCM showed nuclear displacement and were migratory, and only 8% were stationary over a 6-7 h time period (Table 1A). This is similar to the migratory phenotype percentages estimated in the IRM experiments. In contrast, video microscopy of cells treated with REF-CM showed that most cells were not undergoing chemokinesis in response to a stimulus. In addition, differences in mitotic rate were noted. A decreased percentage of HCM-treated cells underwent mitosis. Mitotic activity leads to net nuclear displacement while daughter cells separate and spread (Albrecht-Buehler, 1977a,b), therefore cells observed to undergo mitosis were excluded from tracking data (Table 1B).

Migration rates of REF treated with HCM or REF-CM were determined by calculating the total nuclear displacement for individual cells (Table 1B). Cells cultured in HCM were found to undergo chemokinesis in spurts, moving very quickly (up to 200 μm/h) in one direction, followed by a period of little movement, and then moving very quickly again, usually in another direction. The smaller percentage of REF-CM-treated cells which exhibited some nuclear displacement also tended to locomote sporadically. Both the average migration rate and the average maximal migration rate were significantly higher for HCM-treated cells than

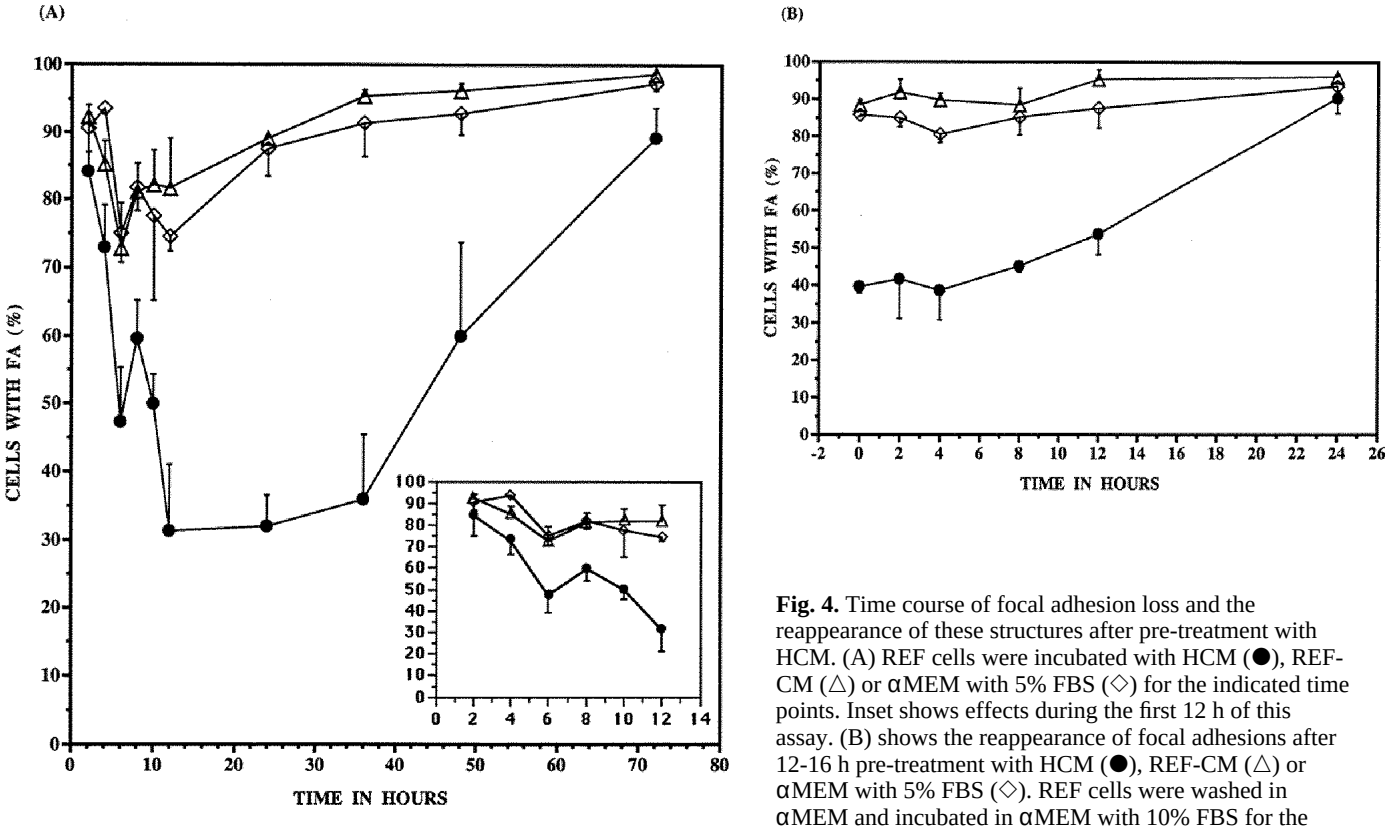

Fig. 4. Time course of focal adhesion loss and the reappearance of these structures after pre-treatment with HCM. (A) REF cells were incubated with HCM (●), REF-CM (△) or αMEM with 5% FBS (◇) for the indicated time points. Inset shows effects during the first 12 h of this assay. (B) shows the reappearance of focal adhesions after 12-16 h pre-treatment with HCM (●), REF-CM (△) or αMEM with 5% FBS (◇). REF cells were washed in αMEM and incubated in αMEM with 10% FBS for the indicated time points so that time point 0 represents the time at which the medium was changed. Data is represented by the percentage of cells with focal adhesions ± s.e.m. in which each point is an average of duplicates for *n*=3 and *n*=2 experiments in (A) and (B) respectively. A different collection of HCM was used in each replicate experiment.

for REF-CM-treated cells. In addition, all cells incubated with REF-CM had at least one period with no nuclear displacement (average minimum 0.0 μm/h). In contrast, the average minimum nuclear displacement of HCM-treated cells was 1.5 μm/h, indicative of more persistent locomotory activity.

Effect of growth factors on focal adhesion presence

Various growth factors were tested for their ability to disperse focal adhesions, in order to determine if one of these molecules is responsible for the migratory effect seen with HCM stimulation. Quantitation of IRM results showed that REF incubated with 20, 2 and 0.2 ng/ml PDGF had 75%, 79% and 81% focal adhesions, respectively. Likewise, 50, 5 and 0.5 ng/ml of either aFGF or bFGF resulted in >84% of the cells containing focal adhesions. Incubation with 25, 5 and 0.5 ng/ml of EGF resulted in 76%, 71% and 76% of cells having focal adhesions present. Lastly, stimulation of REF with 10, 1 and 0.1 ng/ml of TGF-β_1 resulted in >90% of the cells possessing focal adhesions. These results indicate that PDGF, aFGF, bFGF, EGF or TGF-β_1, at the concentrations indicated, could not reduce the percentage of cells with focal adhesions such as was seen with HCM. Currently, efforts are being made to identify the factor(s) which cause a loss of focal adhesions in REF and thereby enhance chemokinesis in these cells.

Time course of focal adhesion loss or assembly

One of the main objectives was to determine how transient the effects of HCM might be and whether its effects were reversible. REF were seeded for 1 h, by which time focal adhesions had not yet formed (data not shown), after which the medium was changed to either HCM, REF-CM or αMEM containing 5% FBS and incubated for up to 72 h. During the first 4 h after the medium change, all samples had a large percentage of the cells containing focal adhesions (Fig. 4A; inset). However, the proportion of HCM-treated cells with focal adhesions decreased after 6 h, while a large proportion of control cells maintained these structures. HCM-treated cells continued to have a lower percentage of cells with focal adhesions than the controls through 48 h (Fig. 4A). By 72 h, all cultures were reaching subconfluency (even though the HCM samples were slower in reaching subconfluency than controls), and all samples contained a high percentage of cells with focal adhesions.

The kinetics of focal adhesion reformation by REF was also examined using the chemokinesis assay, where conditioned medium was removed and replaced with αMEM containing 10% FBS for periods up to 24 h (Fig. 4B). The cells pretreated with HCM maintained a migratory phenotype, with the majority of the cells lacking focal structures, for up to 12 h after the stimulus had been removed, but the effect was lost by 24 h. Cells treated with control media

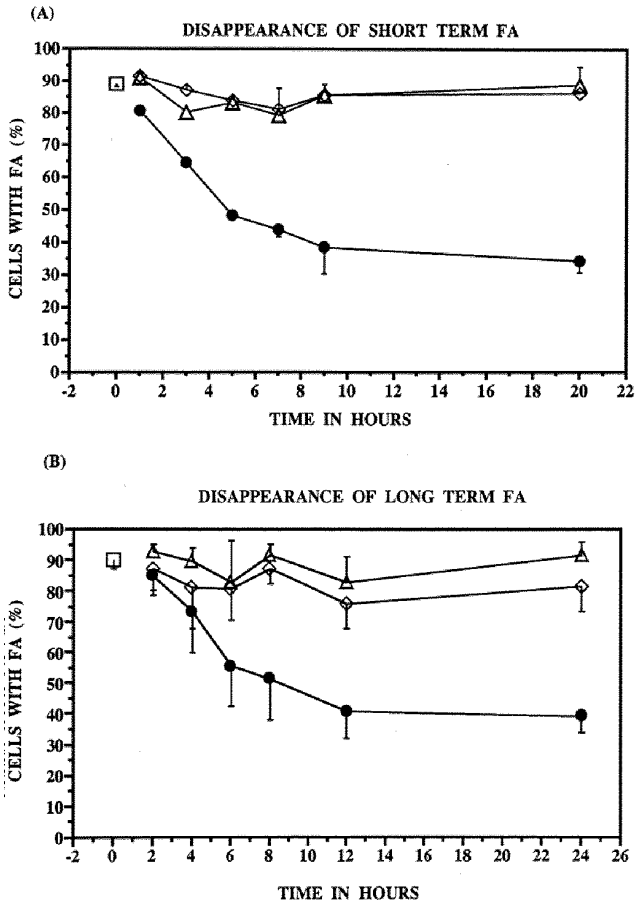

Fig. 5. Disassembly of short and long term focal adhesions. REF cells were allowed to attach, spread and form focal adhesions for 3 h (A), or for 24 h (B). The medium was then switched to HCM (●), REF-CM (△) or αMEM with 5% FBS (◇) and the percentage of cells with focal adhesions ± s.e.m. was observed at the indicated time points. (□) represents the percentage of cells with focal adhesions at the time the medium was changed (time point 0). Points on the graph represent averages of duplicates ± s.e.m. in which n=2 experiments. A different collection of HCM was used in each replicate experiment.

(REF-CM or αMEM containing 5% FBS) maintained a high percentage of cells containing focal adhesions throughout the assay. Therefore, once REF have been stimulated to disperse focal adhesions, the phenotype is maintained for at least 12 h in the absence of HCM.

The ability of HCM to initiate a loss of short and long term focal adhesions was also examined. In the previous experiments, REF cells were allowed to attach and spread for 1 h, after which the medium was removed and replaced with HCM or control media. Although the majority of cells initially assembled focal adhesions and did not subsequently lose them until approximately 6 h later (Fig. 4A), an important parameter was whether REF cells could be stimulated to locomote by adding HCM after the cells had formed focal adhesions under normal growth conditions. As shown in Fig. 5A, REF were allowed to adhere for 3 h, by which time 89% of the cells had focal adhesions. The medium was then changed to HCM, REF-CM or αMEM containing 5% FBS (time point zero), and chemokinesis was estimated by IRM at the indicated time points. Incubation with HCM caused a gradual decrease in the population of cells with focal adhesions, with the majority of the cells exhibiting a complete loss of focal structures at 5-20 h. Other REF cultures were also allowed to form longer term focal adhesions, in which 90% of the cells contained focal adhesions at 24 h. The medium was then changed to HCM or control media, and the percentage of cells containing focal adhesions was determined (Fig. 5B). Six hours after addition of HCM, only 56% of REF still had focal adhesions. At least 45% of the HCM-treated cells exhibited a lack of focal structures from 6-24 h, while most of the control media-treated cells maintained focal adhesions throughout the assay. By comparison with the data shown in Figs 4A and 5A,B, it appears that upon addition of HCM, REF cells require approximately 6 h of lag time before losing their focal adhesions. No difference in lag time was noted between cells that were allowed to attach and spread for 1, 3 or 24 h.

Manipulation of focal adhesions and the cytoskeleton

It was necessary to confirm that alterations in the IRM image were matched by cytoskeletal alterations. REF were allowed to attach and form focal adhesions for 3.5 h, after which growth medium was removed and replaced with HCM. After a 14 h incubation period, HCM was removed, cells were rinsed with PBS and then incubated for an additional 24 h in αMEM containing 10% FBS. Cells were fixed at the indicated time points and stained for F-actin or vinculin (Fig. 6). Focal adhesions originally present at 3.5 h (Fig. 6A-C) were dispersed after 14 h in the presence of HCM (Fig. 6D-F). Consequently, focal adhesions reformed when HCM was removed and replaced with growth medium for 24 h (Fig. 6G-I). Therefore, the system can be used to manipulate focal adhesions and the associated cytoskeleton in a straightforward and predictable manner.

Response of different cell types to HCM

The ability of HCM to stimulate a loss of focal adhesions in a variety of fibroblast cell types was examined to determine if REF were atypical. Two primary cell strains including human embryo fibroblasts (HEF) and human gingival fibroblasts (HGF), and two cell lines, RAT-1 and Balb/c-3T3 cells, were analyzed (data not shown). HEF and Balb/c-3T3 showed no significant difference in the percentage of cells containing focal adhesions between control and HCM-treated cells. Conversely, both HGF and RAT-1 cells responded in a similar way to REF when treated with HCM, in that the percentage focal structures was reduced to <53% and <32% respectively, compared to controls in which the vast majority of cells contained focal adhesions. (Note that the cell lines tested tended to assemble relatively small focal structures compared with the primary cell strains.) Overall, REF seem to be the most responsive to HCM when compared to the corresponding controls.

Effect of HCM on chemotaxis

The ability to stimulate chemokinesis on glass coverslips in cultured primary fibroblast strains is a valuable tool for

the study of cell-substrate interactions, but in addition we also wished to determine whether HCM was capable of stimulating chemotactic migration. Using a modified Boyden chamber, most of the HCM collections were found to stimulate chemotaxis, almost to the same extent as the positive control designed for maximal migration (Fig. 7). The REF-CM did not stimulate greater migration than the FBS control, which consisted of αMEM containing 5% FBS in both the top and bottom chambers. Least migration was detected in the negative control. The results showed that HCM was capable of stimulating both chemokinesis and chemotaxis in REF.

A chemotaxis checkerboard assay was also performed, where various dilutions of HCM and REF-CM were tested.

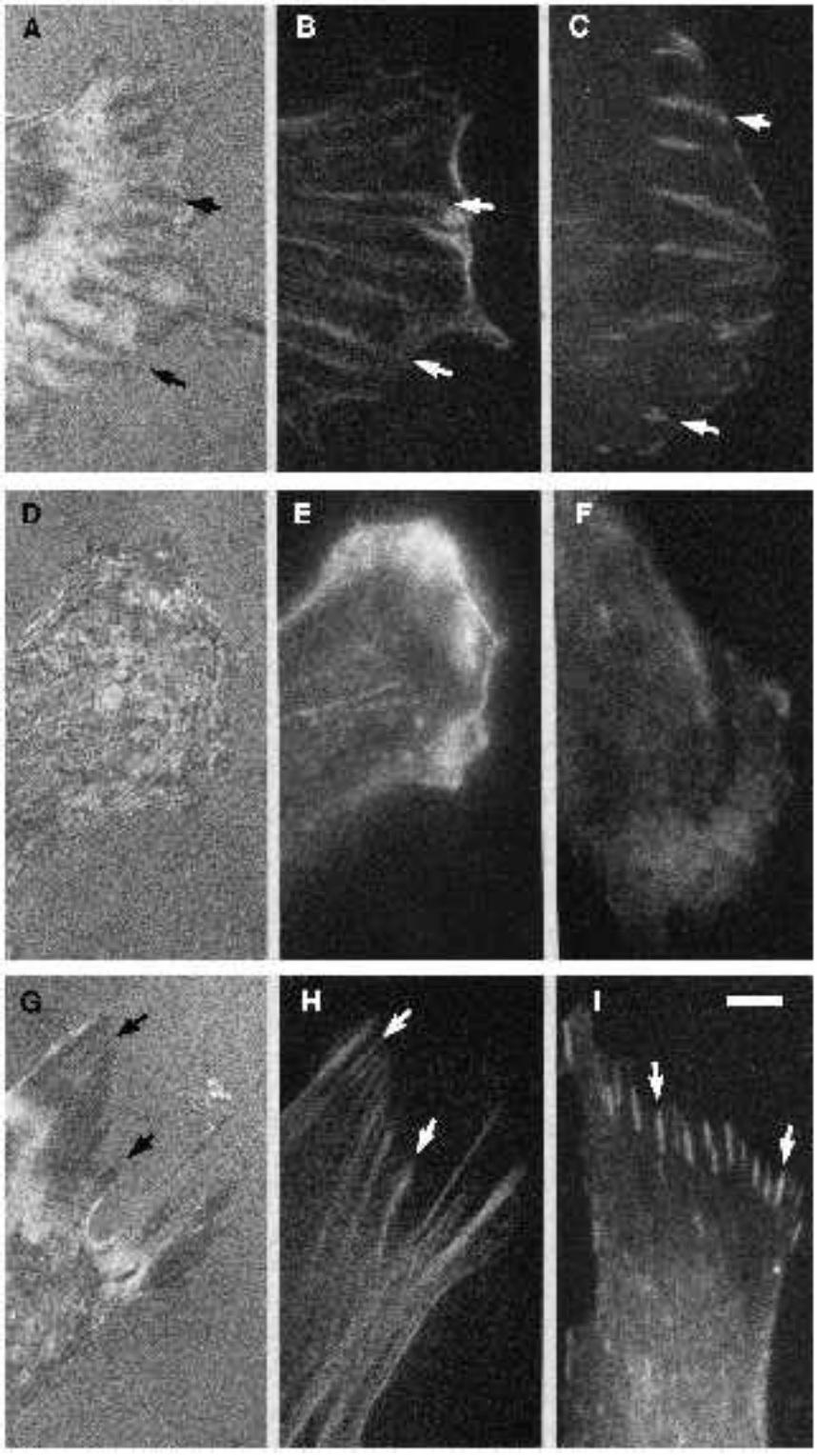

Fig. 6. Manipulation of focal adhesions and the associated cytoskeleton. REF were incubated for 3.5 h in normal growth medium (A-C). HCM was then added to the cells for 14 h (D-F) after which time the stimulus was removed and the cells incubated in growth medium again for 24 h (G-I). IRM micrographs and the corresponding phalloidin staining of F-actin are shown in (A, D, G) and (B, E, H) respectively. Vinculin immunofluorescence is shown in (C, F, I). Focal adhesions and the associated cytoskeleton were manipulated in an on-off manner with focal adhesions, F-actin stress fibers and vinculin plaques present (arrows) at 3.5 h, absent after 14 h in HCM and then present (arrows) again 24 h after removal of the HCM stimulus. Bar, 5 μm.

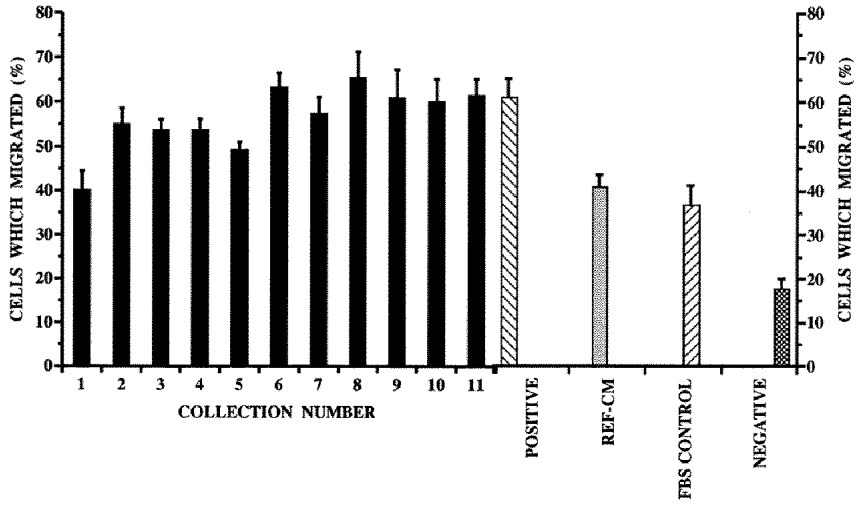

Fig. 7. Chemotaxis stimulated by HCM. REF cells were placed in the top compartment of a modified Boyden chamber with various collections of HCM, (black bars) or control media in the bottom compartment. The positive control (left hatched bar) consisted of 0% FBS in the top and 5% FBS in the bottom compartment. REF-CM is shown by the stippled bar. The FBS control (right hatched bar) consisted of samples with 5% FBS medium in both the top and bottom compartments, and the negative control (checkered bar) consisted of 5% FBS in the top and 0% FBS in the bottom compartment. The cells for the positive control and for the first (serum free) HCM collection were in medium containing 0% FBS, whereas all other samples contained a cell suspension in medium with 5% FBS. Data shown represents the percentage of cells which migrated through the upper 8 μm membrane (as described in Materials and Methods) ± s.e.m. All experimental conditions were performed in duplicate or triplicate; n=6-14 for each HCM sample, n=23 for REF-CM, n=17 and n=18 for positive and negative controls, respectively, and n=8 for FBS control. $P < 0.01$ for HCM collection numbers 2-4 and 6-11 compared to REF-CM.

Since HCM and REF-CM contained FBS, all samples were diluted with αMEM containing 5% FBS. Therefore chemoattractants present in the conditioned medium were effective despite approximately 5% FBS being present in both the top and bottom compartments. In the HCM checkerboard (Table 2A), all dilutions of HCM showed significant chemotactic migration compared with equivalent dilutions of REF-CM (Table 2B). In almost all cases where there was a higher concentration of HCM in the bottom chamber compared to the top chamber (bottom left of diagonal), at least 50% of the REF cells migrated through the upper membrane. However, none of the dilutions of REF-CM resulted in >50% of the cells migrating through the upper membrane. In addition, higher concentrations of REF-CM in the bottom chamber had no effect on migration. Therefore, HCM was effective in stimulating both a chemokinetic and a chemotactic effect in approximately 50% of the cells, even at a 1:20 dilution.

Table 2. Checkerboard assay using various concentrations of HCM or REF-CM

A HCM Checkerboard

Bottom \ Top	αMEM	HCM1:20	HCM1:10	HCM
αMEM	38.5±3.7	36.1±3.7	31.3±7.2	40.4±5.4
HCM1:20	50.0±4.9	37.6±9.0	43.5±4.8	46.4±4.8
HCM1:10	50.4±2.0	56.0±6.7	49.5±3.6	31.3±3.7
HCM	61.7±2.9	49.8±5.1	56.3±4.8	46.0±10.8

B REF-CM Checkerboard

Bottom \ Top	αMEM	REF-CM1:20	REF-CM1:10	REF-CM
αMEM	35.0±8.7	41.5±2.2	47.6±4.2	38.8±5.8
REF-CM1:20	41.2±5.4	43.0±1.8	35.5±9.2	44.9±4.4
REF-CM1:10	44.0±1.9	48.7±11.9	42.4±5.1	38.0±1.5
REF-CM	30.9±4.2	30.9±6.9	18.6.3±5.1	26.1±1.5

Dilutions of HCM (A) and REF-CM (B) in αMEM with 5% FBS were varied in the top and bottom compartments of the modified Boyden chamber. Data represent the average of percentage of cells which migrated through the upper membrane ± s.e.m. in 5 high powered fields (×320). Data were calculated from duplicates or triplicates in which n=4-6. A different collection of HCM was used in each replicate experiment.

Chemokinesis on extracellular matrix substrates

All the experiments above were performed on FBS-coated substrates, but we also determined whether HCM-treatment could affect adhesion in REF plated on several matrix molecules (Fig. 8). In chemokinesis assays, HCM was effective in causing a loss of focal adhesions on murine laminin, bovine plasma fibronectin, the RGD-containing 'cell-binding' domain of plasma fibronectin (105 kDa) and type I collagen (Fig. 8D,F,H,J, respectively). Conversely, control medium-treated cells exhibited many large focal adhesions on all substrates (Fig. 8A,C,E,G,I). The results clearly showed that HCM is capable of stimulating a loss of focal adhesions in REF cells on a number of substrates, whereas under control conditions, cells retained focal structures.

DISCUSSION

The data shown forms the basis for a novel system where the presence of focal adhesions in fibroblasts can be manip-

ulated and locomotion of these cells can also be controlled. Using HCM as the stimulus, a marked loss of focal adhesions in the majority of REF can be induced, resulting in chemokinesis. These effects can be reversed by removal of HCM, so that the presence of focal adhesions and the cytoskeletal components associated with them can be

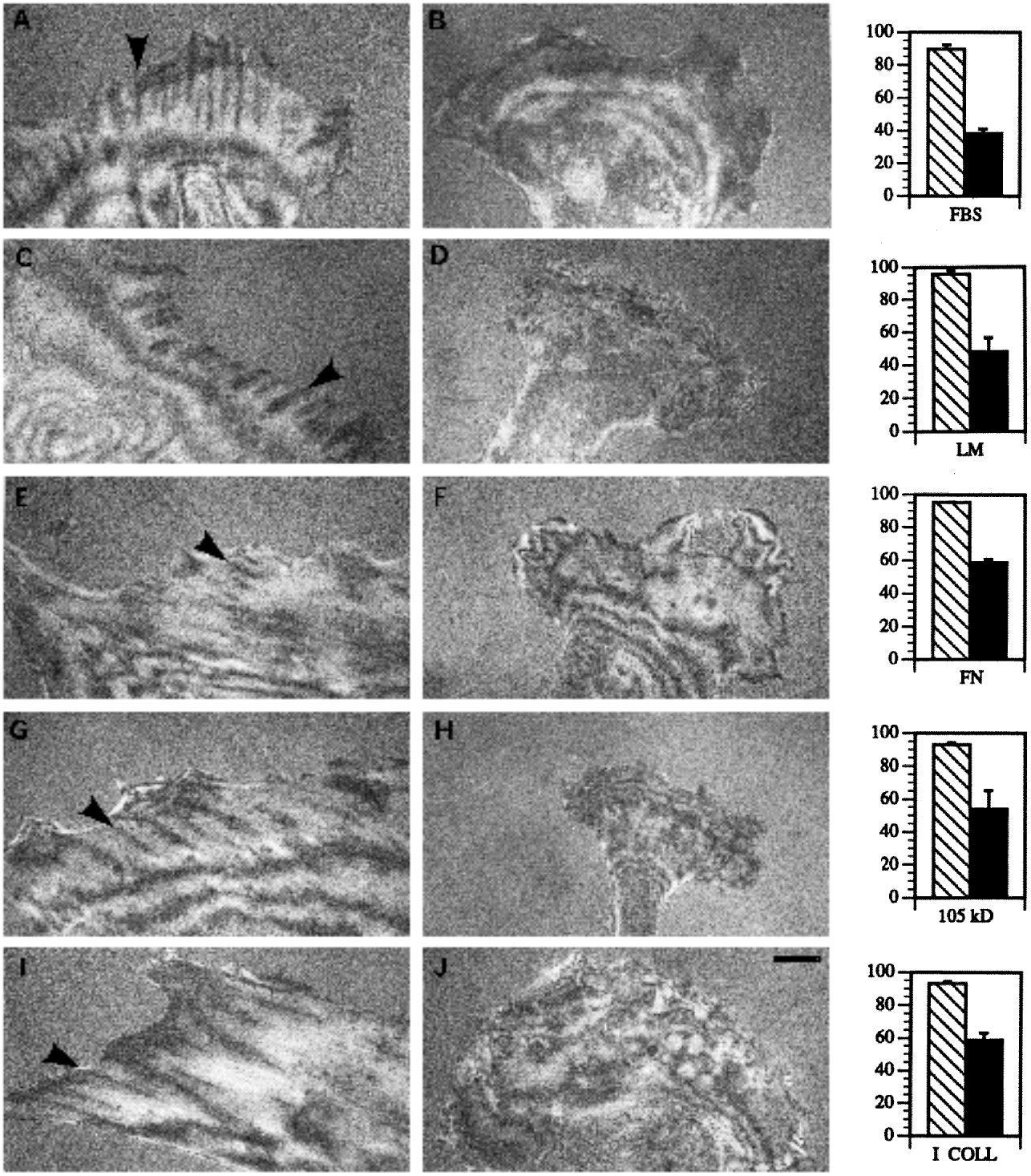

Fig. 8. HCM stimulated a loss of focal adhesions on various extracellular matrix molecules. REF cells were incubated with HCM or REF-CM for 12-16 h on serum (A,B), laminin (C,D), fibronectin (E,F), the 105 kDa 'cell-binding' domain of fibronectin (G,H), and type I collagen (I,J) coated coverslips. (A,C,E,G,I) cells treated with REF-CM exibited a stationery phenotype containing many focal adhesions (arrowheads). However, cells treated with HCM (B,D,F,H,J) lacked focal adhesions and exhibited a migratory phenotype. This experiment was quantitated and the corresponding graphs are shown on the right. REF cells were incubated in REF-CM (hatched bar) or HCM (black bar). Data is represented as the percentage of cells with focal adhesions ± s.e.m.; averages of duplicates from n=2 experiments. Bar, 5 μm.

manipulated in an on-off manner. The factor(s) present in HCM, which are responsible for this effect have yet to be determined. However, several growth factors previously shown to be chemotactic for fibroblasts, i.e. PDGF, EGF, aFGF, bFGF and TGF-β_1, have been shown here to cause no significant decrease in the percentage of cells containing focal adhesions. Currently, efforts are being made to isolate and identify factor(s) responsible for the loss of focal adhesions and concomitant stimulation of locomotion.

The factor(s) in HCM which are responsible for enhancing locomotion are probably secreted by the highly migratory fibroblasts moving from the minced heart tissue. Therefore, it appears that 'wounded' connective tissue fibroblasts release autocrine locomotion-enhancing factors which can be used to stimulate cultured primary fibroblasts to migrate in vitro. Grey et al. (1989) have isolated a migration stimulating factor (MSF) from fetal and breast cancer patient fibroblasts which induces chemotactic migration of adult fibroblasts (Schor et al., 1988a,b). However, no effects of MSF on focal adhesions have been reported. A similarity with our system is that pre-treatment with HCM or MSF can induce prolonged migration in the absence of the factor(s) (Schor et al., 1989). Whether MSF is present in HCM and is responsible for the loss of focal adhesions resulting in chemokinesis remains to be resolved. It is also unlikely that hepatocyte growth factor/scatter factor (HGF/SF) is responsible for the locomotion-enhancing effect since HGF/SF has been previously shown to have no noticeable effects on fibroblast movement or morphology (Stoker et al., 1987).

In other studies, HCM has been found to be a potent inducer of neurite outgrowth (Collins, 1980). HCM, used as a substrate, was found to mediate adhesion and migration of neuronal growth cones in an arginine-glycine-aspartic acid (RGD)-independent, but β_1 integrin-dependent, manner (Letourneau et al., 1988). HCM appears to be most potent in stimulating neuronal migration when used as a substrate in a model of haptotaxis (Collins and Lee, 1984). However, the effects of HCM on non-neuronal cells had not been previously examined.

We utilized a chemokinesis assay in which locomotion was estimated by determining the proportion of cells which lacked focal adhesions. Previous studies have shown that highly locomotory cells, with motility rates up to 80 μm/h, lacked focal adhesions; instead these cells were characterized by the presence of close contacts (Izzard and Lochner, 1976; Couchman and Rees, 1979b). Therefore, in the system presented here, the lack of focal adhesions was used as an index for estimating the proportion of cells undergoing locomotion. Video microscopy experiments confirmed that HCM was actually stimulating chemokinesis and not merely a loss of focal adhesions, as seen by IRM. REF cells were estimated to locomote at rates up to 200 μm/h, with an average maximal migration rate in HCM-stimulated cells of 140 μm/h. In addition to stimulating chemokinesis, HCM was found to be a potent inducer of chemotaxis. During the chemotaxis experiments, serum was present in both the top and bottom compartments of modified Boyden chambers, thus HCM had to override the presence of previously characterized chemotactic growth factors, such as PDGF (Grotendorst, 1988), in order to exhibit a migration-enhancing effect. Therefore HCM was shown to have considerable potency, and this was confirmed by the checkerboard assays.

HCM-treated cells which lacked focal adhesions when examined by IRM also lacked F-actin stress fibers, and had vinculin and talin localized in a diffuse pattern. These cells often had a concentration of F-actin and talin but not vinculin in the leading lamella, consistent with previous studies on locomotory cells (Small and Langanger, 1981; Burridge and Connell, 1983; DePasquale and Izzard, 1991).

Of various cell types tested, REF had the largest response towards HCM, although HCM treatment was also found to decrease the proportion of HGF containing focal adhesions. Of the two cell lines tested, RAT-1 cells had the best response in terms of loss of focal structures. However, one of the main objectives of this study was the development of a model for controlled migratory activity in primary fibroblast strains which might more closely resemble the in vivo state.

HCM stimulated a loss of focal structures on a variety of substrates. The fibronectin used in these experiments was isolated from bovine plasma, and previous results have shown that fibroblasts migrating from explants require both endogenous and exogenous fibronectin for locomotion (Couchman et al., 1982). Furthermore, ffrench-Constant et al. (1989) have shown that different alternatively spliced forms of fibronectin are present at different stages during wound healing. Matrix deposition and modification by HCM-treated cells may therefore be an important issue for future study.

In conclusion, we report the first system where the initiation and subsequent termination of migration in fibroblast populations can be readily controlled. This model utilizes autocrine or paracrine factors which stimulate a loss of focal adhesions and persistent chemokinetic and chemotactic activity. This system is independent of extracellular matrix substrates, and is predictable, reproducible and may closely resemble the response of cells to tissue injury. Efforts to understand the molecular basis of focal adhesion assembly and disassembly, recently the focus of much research (Guan et al., 1991; Burridge et al., 1992; Damsky and Werb, 1992; Guan and Shalloway, 1992; Ridley and Hall, 1992; Ridley et al., 1992; Woods and Couchman, 1992), may be considerably aided by this straightforward system. While migration of connective tissue cells is a corollary of development, wound repair and in some cases metastatic behavior, the control of switching between migratory and anchored phenotypes is not understood. The results described here may provide a framework for studies, at many levels, on the regulation of cell locomotion.

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