

Thrombospondin signaling through the calreticulin/LDL receptor-related protein co-complex stimulates random and directed cell migration

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Summary

The matricellular extracellular matrix protein thrombospondin-1 (TSP1) stimulates focal adhesion disassembly through a sequence (known as the hep I peptide) in its heparin-binding domain. This mediates signaling through a receptor co-complex involving calreticulin and low-density lipoprotein (LDL) receptor-related protein (LRP). We postulate that this transition to an intermediate adhesive state enhances cellular responses to dynamic environmental conditions. Since cell adhesion dynamics affect cell motility, we asked whether TSP1/hep I-induced intermediate adhesion alters cell migration. Using both transwell and Dunn chamber assays, we demonstrate that TSP1 and hep I gradients stimulate endothelial cell chemotaxis. Treatment with focal adhesion-stabilizing concentrations of TSP1/hep I in the absence of a gradient enhances endothelial cell random migration, or chemokinesis, associated with an increase in cells migrating, migration speed, and total cellular

displacement. Calreticulin-null and LRP-null fibroblasts do not migrate in response to TSP1/hep I, nor do endothelial cells treated with the LRP inhibitor receptor-associated protein (RAP). Furthermore, TSP1/hep I-induced focal adhesion disassembly is associated with reduced chemotaxis to basic fibroblast growth factor (bFGF) but enhanced chemotaxis to acidic (a)FGF, suggesting differential modulation of growth factor-induced migration. Thus, TSP1/hep I stimulation of intermediate adhesion regulates the migratory phenotype of endothelial cells and fibroblasts, suggesting a role for TSP1 in remodeling responses.

Movies available online

Key words: Thrombospondin-1, Focal adhesion, Cell migration, Calreticulin, LDL receptor-related protein

Introduction

Integrin-mediated adhesions are heterogeneous structures displaying varying degrees of organization, culminating in the focal adhesion plaque. Focal adhesions consist of integrin clusters linked to bundled actin microfilaments, termed stress fibers, through anchor proteins, such as talin, vinculin and α -actinin. Mostly found in highly adherent cells, such as endothelial cells and fibroblasts, focal adhesions are thought to indicate a highly stable interaction between the cell and the extracellular matrix (ECM) (Couchman and Rees, 1979). However, the function of these structures in cellular physiology and the mechanisms regulating their dynamics remain poorly defined. (Zamir and Geiger, 2001; Adams, 2002).

Cell migration is an integral process in tissue formation and remodeling, and is dependent upon adhesion dynamics (Kaverina et al., 2002; Webb et al., 2002). Nascent adhesions form in the leading lamellipodia, stabilizing the protrusion and generating traction to pull the cell body forwards (Beninger et al., 2001). However, adhesions in the rear of the cell must disassemble to allow retraction of the trailing edge of the cell. While focal adhesion plaques may provide some of the

tractional forces driving cell migration, the relative stability of focal adhesions, compared with less-organized adhesive structures, may retard rear retraction (Lauffenburger and Horwitz, 1996; Webb et al., 2002). Increased expression of vinculin and α -actinin enhances focal adhesion formation and reduces cell migration, whereas decreased expression reduces focal adhesion formation and stimulates migration (Fernandez et al., 1992; Gluck and Ben-Ze'ev, 1994). Several pro-migratory signaling pathways, such as phosphoinositide 3-kinase (PI 3-kinase), src and focal adhesion kinase (FAK), also regulate focal adhesion dynamics (Greenwood and Murphy-Ullrich, 1998; Anand-Apte and Zetter, 1997). Thus, modulating focal adhesion structure, by altering either the expression of certain focal adhesion components or the signaling pathways that regulate their assembly, significantly affects cell migration.

Thrombospondin-1 (TSP1) is a large, homotrimeric, matricellular glycoprotein, expressed in a highly regulated manner by numerous cell types in developing and remodeling tissues. TSP1 is involved in numerous biological functions, probably attributable to its multiple domains and cell-surface

receptors as well as its ability to act as either a soluble or matrix-bound factor. Multiple domains of TSP1 affect cell migration, although regulation of domain dominance remains poorly understood and probably depends on the cell type and environmental context. Matrix-bound TSP1 supports a limited degree of cell attachment and spreading, characterized by the formation of fascin microspikes in the cell periphery and the absence of focal adhesions or stress fibers (Adams, 1995; Murphy-Ullrich and Höök, 1989). Haptotactic migration to matrix-bound TSP1 occurs through the pro-adhesive C-terminal domain, although other domains might also be involved (Taraboletti et al., 1987). The heparin-binding domain (HBD) elicits cell migration in neutrophils, monocytes, melanoma cells and endothelial cells, and is suggested to mediate TSP1-induced chemotaxis (Mansfield et al., 1990; Mansfield and Suchard, 1994; Taraboletti et al., 1987; Taraboletti et al., 1990). However, the mechanisms by which the HBD stimulates cell migration are not established.

TSP1 destabilizes cell-ECM adhesions by stimulating focal adhesion disassembly in highly adherent cells and preventing focal adhesion formation in adhering cells. TSP1-induced focal adhesion disassembly involves unbundling of actin stress fibers and the selective depletion of vinculin and α -actinin from the focal adhesion plaque (Greenwood et al., 1998; Greenwood and Murphy-Ullrich, 1998). This transition does not affect integrin clustering or cell spreading, representing a reversion from a mature focal adhesion to a less-organized adhesive structure. This spread cell depleted of focal adhesions is termed the intermediate adhesive state by our group, and is postulated to prime the cell for dynamic cellular processes (Greenwood and Murphy-Ullrich, 1998; Murphy-Ullrich, 2001). TSP1 stimulates the transition to intermediate adhesion through a 19-amino acid sequence (hep I peptide) in the HBD, which signals focal adhesion turnover through a receptor co-complex of calreticulin (CRT) and low-density lipoprotein (LDL) receptor-related protein (LRP) (Murphy-Ullrich et al., 1993; Goicoechea et al., 2000; Orr et al., 2002; Orr et al., 2003). We now report that TSP1/hep I stimulates chemotaxis and chemokinesis in endothelial cells and fibroblasts through ligation of the CRT-LRP receptor complex, and selectively modulates fibroblast growth factor (FGF)-induced migration.

Materials and Methods

Materials

The following items were utilized: Dulbecco's modified Eagles medium (DMEM; Cell-Gro, Mediatech, Herndon, VA), fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 500 μ g/ml trypsin, 2 mM EDTA (Life Technologies, Grand Island, NY). Fibronectin was from Becton Dickinson Biosciences (Bedford, MA) and from Sigma-Aldrich (St Louis, MO). Vitronectin was from Biosource International (Camarillo, CA). Calcein AM was from Molecular Probes (Eugene, OR).

Proteins

TSP1 was isolated from human platelets from the American Red Cross, and purified as previously described using heparin affinity and gel filtration chromatography (Murphy-Ullrich et al., 1993). Peptides hep I (ELTGAARKGSGRRLLVKGPDG) and modified hep I (ELTGAARAGSGRRLLVAGPDG) were synthesized, purified and analyzed by the University of Alabama at Birmingham

Comprehensive Cancer Center/Peptide Synthesis and Analysis shared facility and by AnaSpec (San Jose, CA). Receptor-associated protein (RAP) was a generous gift of Dudley Strickland (Jerome Holland Labs, ARC, Bethesda, MD). Bovine bFGF and aFGF were from Calbiochem (San Diego, CA).

Cell culture

Bovine aortic endothelial (BAE) cells were isolated and cultured in DMEM containing 4.5 g/l glucose, 2 mM glutamine and 10% FBS as described previously (Murphy-Ullrich et al., 1993). Wild-type (K41) and CRT-null (K42) mouse embryonic fibroblasts (MEFs) were a gift of Marek Michalak (University of Alberta, Edmonton, AB, Canada). LRP-null [PEA 13 (ATCC-CRL-2216)] MEFs were from American Type Culture Collection (Manassas, VA). Growth conditions for MEFs were the same as described for BAE cells.

Transwell assay

A 96-well chemotaxis chamber (Neuro Prob, Gaithersburg, MD) was used for the transwell assays. BAE cells were grown to near confluence and fluorescently labeled by incubation with 1 μ M calcein AM for 10 minutes. Cells were washed twice in DMEM and briefly trypsinized for re-plating on the transwell membrane. The polyvinyl membrane containing 8 μ m pores was coated for 5 hours with 10 μ g/ml vitronectin and 10 μ g/ml fibronectin, washed three times in phosphate buffered saline (PBS) and air-dried. Increasing concentrations of hep I, TSP1, modified hep I peptide or bFGF were added to the lower chamber, and the membrane was fastened to the lower chamber per manufacturer's instructions. Labeled BAE cells were then plated on the top side of the filter at 2×10^4 cells per well in either serum-free media or in serum-free media containing increasing concentrations of hep I, TSP1, modified hep I or bFGF. Cells were allowed to migrate towards the bottom well for 5 hours. The top side of the membrane was scraped using pre-wetted cotton swabs and gently rinsed with PBS. The chamber was loaded into a plate reader (emission 460 nm/absorbance 530 nm) and the remaining well-associated fluorescence was read. Results for each assay were carried out in triplicate and normalized to migration levels seen with serum-free media alone.

Dunn chamber

Dunn chambers were from Weber Scientific International (Teddington, UK). The Dunn chamber allows for generation of a stable chemotactic gradient and observation of cell migration in the context of the gradient (Zicha et al., 1991). Glass coverslips were coated with 10 μ g/ml fibronectin and 10 μ g/ml vitronectin for 5 hours. Cells were then sparsely plated onto the pre-coated coverslips and allowed to attach in serum-free media for 3 hours. Serum-free DMEM containing 23.8 mM HEPES, with or without 100 nM hep I, 7.8 nM TSP1 or 100 nM modified hep I, was added to both Dunn chamber wells. Glass coverslips were then loaded onto the Dunn chamber, cells down, and sealed to the Dunn chamber by an equal mixture of vacuum grease and Vaseline (blotted to remove excess oil), with the outer well of the Dunn chamber remaining uncovered. Media was removed from the outer well, and serum-free DMEM containing 23.8 mM HEPES, with or without 100 nM hep I, 7.8 nM TSP1, 100 nM modified hep I, 0.1% FBS, 61 pM bFGF or 67 pM aFGF, was added to the outer well, establishing a concentration gradient. A computer-controlled stage (Prior Scientific, Rockland, MA) was used to enable viewing of multiple fields on each of two Dunn chambers over the time course of the experiment using software developed in the Dennis Kucik laboratory. Cells were imaged on an Axiovert 100 microscope (Zeiss, Thornwood, NY) equipped with a CCD camera (Model 300T-RC, Dage-MTI, Michigan City, IN). Temperature on the stage was kept constant at 37°C, and images of each field were captured at 2 minute-

intervals for a total of 6 hours and 40 minutes (200 frames) using a WinTV video card (Hauppauge Computer Works, Hauppauge, NY) and software written in the Kucik laboratory. Resulting time-lapse video was analyzed by Metamorph software (Universal Imaging Corporation, Downingtown, PA) to track individual cell migration paths through successively calculating (x,y) coordinates of the centroid of the cell. Slight movements in position resulting from the use of a movable stage were removed by simultaneous tracking of a stationary point, and normalization of the resulting (x,y) coordinates generated by Metamorph. Tracks were then analyzed using programs written by Dennis Kucik for this purpose. Total track distance is determined as the sum of the incremental distances between successive (x,y) coordinates. Cell speed was determined as the total distance migrated divided by the time of the assay (generally 400 minutes). The total cellular displacement is calculated as the distance between the final (x,y) coordinate and the initial (x,y) coordinate. For each cell, x-displacement and y-displacement is also calculated using this method, and can be used as final cell position given an initial cell position at the origin (0,0).

Statistical analysis

Statistical significance was determined using Student's unpaired *t*-tests and analysis of variance (ANOVA). Results were considered to be significant at $P < 0.05$ or $P < 0.01$.

Online supplemental material

BAE and MEF cell migration in the Dunn chamber was recorded at 2-minute intervals over 6 hours and 40 minutes (200 frames) using the WinTV video card. Time-lapse videos were then created using Metamorph software. Videos were recorded at 30 frames per second and compressed using the Cinepak codec. Movie 1 depicts BAE cell migration under serum-free conditions, whereas Movie 2 illustrates chemokinetic migration in BAE cells treated with hep I (100 nM). Movie 3 illustrates the migratory defect in LRP-knockout MEFs under serum-free conditions. Movie 4 shows chemotactic migration towards an aFGF (67 pM) gradient, with the gradient highest on the right side of the screen, whereas Movie 6 depicts the same aFGF-induced chemotaxis in the presence of chemokinetic concentrations of hep I (100 nM). Movie 5 represents chemotactic migration towards a bFGF (61 pM) gradient on the left side of the screen, whereas Movie 7 illustrates the effect of chemokinetic hep I (100 nM) treatment on bFGF-induced chemotaxis.

Results

TSP1/hep I stimulates endothelial cell migration in the transwell assay

The transwell assay for quantifying cell migration measures the migration of cells across a porous membrane in response to potential migratory stimuli. This assay allows for simultaneous testing of multiple conditions, making it ideal to screen for potential migratory stimuli or to determine dose dependency of migration responses. However, the transwell assay only measures positive and negative responses, limiting the scope of information provided. BAE cells were loaded with the fluorescent dye calcein AM, plated onto the porous transwell membrane, and stimulated with increasing concentrations of TSP1 on the other side of the membrane. TSP1 induced a dose-dependent increase in BAE cell migration towards TSP1 in the lower well (Fig. 1A). This effect was maximal at 1 $\mu\text{g/ml}$ (7.8 nM monomer), and higher concentrations resulted in decreased cell migration. TSP1 induced chemotaxis to a similar extent as bFGF (61 pM), suggesting the potency of this chemotactic

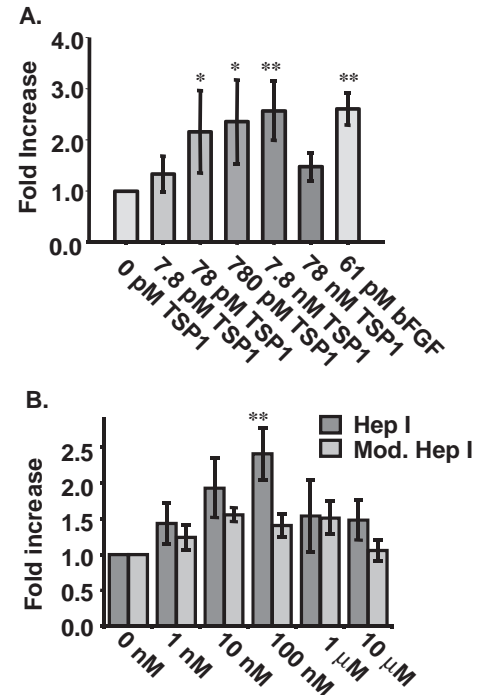


Fig. 1. TSP1/hep I stimulate directed endothelial cell migration. (A) BAE cells were grown to near confluence, loaded with calcein AM, and plated onto a 96-well Neuro Probe chemotaxis chamber coated with vitronectin and fibronectin. Cells were stimulated to migrate towards increasing concentrations of soluble TSP1 for 5 hours. As a positive control, cells were also stimulated to migrate towards a bFGF gradient. Cells were scraped from the upper surface and cells migrating to the lower surface assayed by determining the remaining fluorescence in a plate reader. Migration was normalized to that seen with media alone and presented as fold stimulation of migration above baseline levels. $n=4-6$, * $P < 0.05$, ** $P < 0.01$. (B) Cells were stimulated to migrate towards increasing concentrations of either the hep I peptide or a modified, inactive hep I peptide, and migration was assessed as described in (A). $n=3$, ** $P < 0.01$.

response is relevant to endothelial cell function. In addition, increasing concentrations of the focal adhesion-labilizing hep I peptide stimulated endothelial cell migration to a similar extent as intact TSP1 (Fig. 1B). An inactive, modified hep I peptide, containing alanine residues in place of two essential lysines, did not stimulate endothelial cell migration. The maximal migration response to the hep I peptide occurred at 100 nM and, like TSP1, the effect of hep I on endothelial cell migration was reduced at higher concentrations. This effect might result from saturation of available receptors, inhibiting the ability of the cell to sense the gradient, as occurs with most growth factors.

Since focal adhesion disassembly potentially affects the ability of cells to migrate, we assessed the effect of hep I on random endothelial cell migration (chemokinesis) by incubating cells with increasing concentrations of hep I on both sides of the transwell membrane. Increases in chemokinetic migration will result in increased movement of endothelial cells to the lower surface of the transwell membrane despite the lack of a chemical gradient directing the response. Increasing concentrations of hep I in both the upper and lower

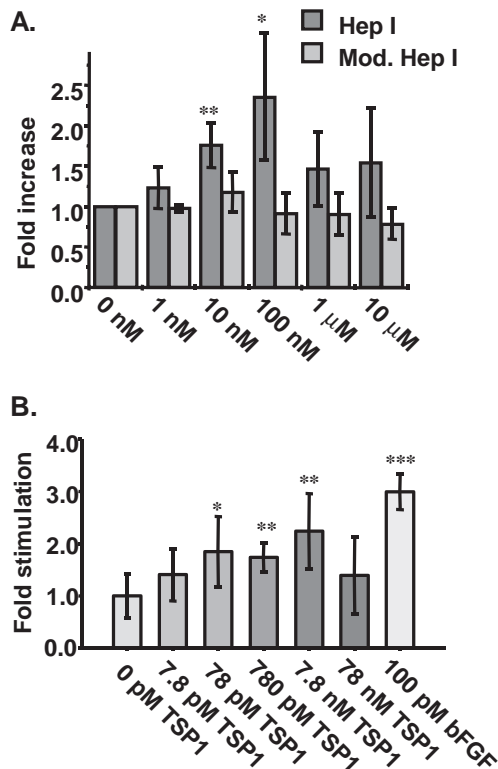


Fig. 2. Hep I increases random migration in endothelial cells. (A) BAE cells were grown to near confluence, loaded with calcein AM, and plated onto a 96-well Neuro Probe chemotaxis chamber coated with vitronectin and fibronectin. Cells were stimulated to migrate in response to increasing concentrations of hep I, or the inactive, modified hep I peptide, in the absence of a gradient for 5 hours. Cells were scraped from the upper surface and cells migrating to the lower surface assayed by determining the remaining fluorescence in a plate reader. Migration was normalized to that seen with media alone and presented as fold stimulation of migration above baseline levels. $n=3-4$, $*P<0.05$, $**P<0.01$. (B) Cells were stimulated to migrate with increasing concentrations of TSP1 in the absence of a gradient and migration was assessed as described in (A). Treatment with 100 pM bFGF was included as a positive control in this assay. $n=3$, $*P<0.05$, $**P<0.01$.

wells stimulate an increase (~2.5-fold) in the random motility of endothelial cells (Fig. 2A), and this response, like hep I-induced chemotaxis, was also maximal at 100 nM hep I peptide. This concentration of hep I is also sufficient to stimulate maximal focal adhesion turnover, suggesting a correlation between the presence of focal adhesions and the ability of endothelial cells to move (Murphy-Ullrich et al., 1993). The modified hep I peptide did not affect endothelial cell migration over the range of concentrations tested. As positive controls, both TSP1 and bFGF were tested for the ability to stimulate endothelial cell chemokinesis. Like TSP1-induced chemotaxis, TSP1 stimulates a dose-dependent increase in endothelial chemokinesis (~2-fold) that is maximal at 7.8 nM monomer concentration (Fig. 2B) and, similar to hep I-induced chemokinesis, this concentration is maximal for induction of focal adhesion disassembly by TSP1. Consistent with previous reports, bFGF (100 pM) stimulated a strong increase in endothelial cell chemokinesis (~3-fold).

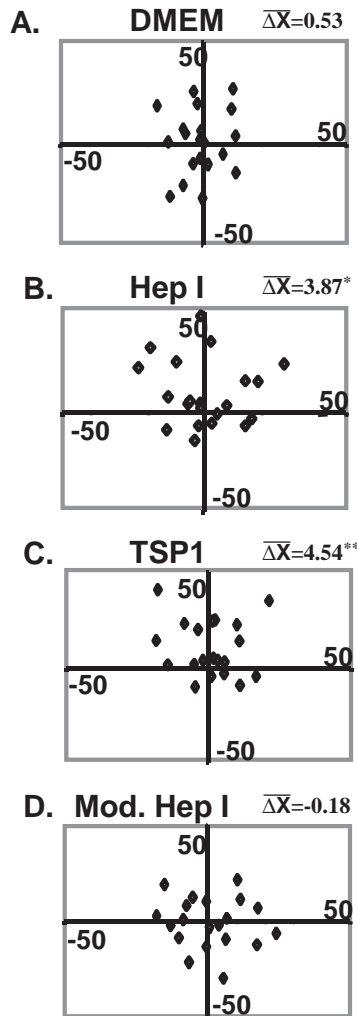
Viewing TSP1/hep I-induced migration in the Dunn chamber

The Dunn chamber, named for and developed by Graham Dunn, is a modified helicobacter counting chamber capable of establishing chemical gradients across a bridge region between an inner circular well and an outer concentric well (Zicha et al., 1991). Cells are plated onto glass coverslips and mounted on the chamber in the presence of varying stimuli. The chamber is sealed, and the cells over the bridge region monitored by time-lapse videomicroscopy. These videos can then be analyzed by cell-tracking software to quantify the orientation, directionality, distance and speed of the cells migrating over this bridge region. BAE cell migration in response to serum-free DMEM or serum-free DMEM with a TSP1 (7.8 nM), hep I (100 nM) or modified hep I (100 nM) gradient was recorded, and individual cell migration tracks were determined. At least 20 cells were analyzed per field, and 3-6 fields were analyzed per condition (60-120 cells total). A representative scatter plot is shown for each condition, with the starting point of the cell designated as the origin of the graph and the top of the graph representing the outer well or the source of the gradient. In addition, the average distance migrated towards the outer well is also shown. Whereas endothelial cell migration is not directionally oriented under serum-free conditions (Fig. 3A), the addition of either TSP1 or hep I ($P<0.01$ and $P<0.05$, respectively) to the outer well induced a markedly directional response (Fig. 3B,C). By contrast, the inactive modified hep I peptide did not orient BAE cell migration (Fig. 3D). Chemotactic concentrations of TSP1/hep I resulted in a slight, but insignificant, increase in BAE cell migration distance, speed and displacement (data not shown). These data suggest that TSP1/hep I stimulates a chemotactic response in endothelial cells and that migration in response to TSP1/hep I gradients in the transwell probably reflects chemotactic migration.

Increased chemokinesis following TSP1/hep I treatment

The Dunn chamber can also be used to assess chemokinesis by observing general migratory parameters, such as migration distance, speed, displacement and directionality, in cells stimulated in the absence of a chemical gradient. BAE cells were incubated in serum-free DMEM with or without TSP1 (7.8 nM), hep I (100 nM) or modified hep I (100 nM), in both the inner and outer wells of the Dunn chamber, and migration parameters were determined as described in the Materials and Methods section. TSP1 and hep I induce endothelial cell chemokinesis, characterized by an increase in the average cell migration speed, the total cellular displacement and the percent of migrating cells (Table 1; Movies 1 and 2 available at jcs.biologists.org/supplemental). The modified hep I peptide did not affect general migration parameters as compared with media alone. As expected, TSP1/hep I-induced chemokinetic migration did not show a preference for migration orientation, unlike chemotactic migration towards TSP1/hep I (data not shown). Migration directionality is measured by dividing the total distance of cell migration by the resultant vector or total displacement (Noble and Levine, 2000). Under this method, a purely directional migration track will show a ratio of cellular displacement to total migration distance equal to 1, meaning every step was taken in the exact same direction. Chemokinetic

Fig. 3. TSP1/hep I-induced endothelial cell chemotaxis in the Dunn chamber. BAE cells were plated at low density under serum-free conditions onto glass coverslips coated with vitronectin and fibronectin. Cells were allowed to attach for 3 hours and loaded onto the Dunn Chamber in serum-free media. Serum-free media was removed from the outer well and media containing either serum-free media (A), hep I (100 nM) (B), TSP1 (7.8 nM) (C) or modified hep I (100 nM) (D) was added to the outer well. The chamber was sealed, and time-lapse video was taken of the cells over a 7-hour time span. Migration of individual cells was tracked using Metamorph software and individual tracks were analyzed for distance, displacement, directionality and orientation. Final positions of the cells are plotted with the initial point being the origin, and the top of the graph representing the outer well. Results are representative of at least three separate experiments. Average displacement ($\overline{\Delta X}$) toward the outer well is given for each treatment. * $P < 0.05$, ** $P < 0.01$.



TSP1/hep I stimulated a slight, but insignificant, increase in BAE cell migration directionality. Thus, TSP1/hep I enhances the percent of cells migrating and speed of cell migration, without appreciably changing migration directionality, resulting in a net increase in cellular displacement.

The apparent increase in average cell migration speed could occur either through stimulation of migration in a subpopulation of nonmigratory cells or through more-subtle alterations in migration speed over the cell population. To determine how TSP1/hep I affects migration speed within the BAE cell population, histograms of cells migrating at certain speed intervals in response to basal or TSP1/hep I-stimulated conditions were produced. Cells migrating under serum-free conditions display a Gaussian distribution of average cell migration speeds, with most cells migrating in the 0.03–0.09 $\mu\text{m}/\text{minute}$ range (Fig. 4A). Treatment with TSP1 and hep I induces an increase in the percent of cells migrating at high speeds, while decreasing the percent of cells migrating at low speeds (Fig. 4B,C). This effect was not seen with cells treated with the inactive modified hep I peptide (Fig. 4D). The population of endothelial cells, both treated and untreated, displayed a wide range of average cell speeds, suggesting varying degrees of locomotion without clear distinctions

Table 1. Endothelial migration parameters following TSP1/hep I treatment

Treatment	Migration speed ($\mu\text{m}/\text{minute}$)	Displacement (μm)	Directionality (displacement/distance)	Percent migrating (speed > 0.08)
DMEM	0.066 \pm 0.009	11.1 \pm 2.23	0.391 \pm 0.101	34.6 \pm 8.50
Hep I	0.101 \pm 0.006	18.0 \pm 2.21	0.509 \pm 0.069	67.2 \pm 16.6
TSP1	0.104 \pm 0.014	18.4 \pm 0.075	0.434 \pm 0.051	71.3 \pm 13.3
Modified hep I	0.065 \pm 0.023	11.5 \pm 3.84	0.418 \pm 0.038	31.2 \pm 24.6

between migrating and non-migrating cells. Whereas activation of a subpopulation of cells would have resulted in a biphasic distribution of cell speeds, TSP1/hep I treatment increased migration speed across the entire population of endothelial cells.

CRT and LRP mediate TSP1/hep I-induced cell migration

TSP1 signals focal adhesion disassembly through interactions between the hep I sequence of TSP1 and cell-surface CRT (Goicoechea et al., 2000). Following hep I binding, the interaction between cell-surface CRT and LRP is altered, resulting in activation of a downstream signaling cascade culminating in focal adhesion disassembly. TSP1/hep I does not stimulate focal adhesion disassembly or activation of downstream signaling pathways in fibroblasts derived from either CRT-knockout or LRP-knockout mice, making these cells useful tools for studying the specificity of this response (Goicoechea et al., 2002; Orr et al., 2002) (Orr et al., 2003).

The ability of TSP1/hep I to induce chemokinesis in wild-type and CRT-knockout fibroblasts was determined through Dunn chamber analysis. Wild-type fibroblasts demonstrated increased cell displacement (R) following TSP1/hep I treatment, illustrated by scatter plots of the final cell position (Fig. 5A,C,E). However, fibroblasts derived from CRT-knockout mice did not show increased migration in response to TSP1/hep I treatment (Fig. 5B,D,F). Both cell lines migrated similarly in response to 0.1% FBS (Fig. 5G,H), indicating that CRT-knockout fibroblasts are not migration deficient, but specifically fail to respond to TSP1/hep I. A closer look at the TSP1/hep I-induced changes in cell migration revealed a disparity in TSP1/hep I-induced cell migration between endothelial cells and fibroblasts. TSP1 and hep I stimulate migration to a similar extent in endothelial cells (Table 1). However, fibroblasts show different responses depending on whether they are stimulated with TSP1 or the hep I peptide. Treatment with hep I stimulates increased migration speed, total displacement and the percentage of migrating cells, similar to that seen in endothelial cells treated with TSP1/hep I (Fig. 6A–C). However, in fibroblasts, TSP1 did not stimulate as significant an increase in migration speed or the percent of migrating cells, but had a profound effect on migration directionality (Fig. 6A,B,D). However, the total cell displacement induced by TSP1 and hep I in the fibroblasts was similar with either treatment (Fig. 6C). The ability of TSP1 to act differently to hep I alone indicates that TSP1 affects fibroblast migration through other domains and receptors as well. However, the CRT-knockout fibroblasts showed impaired TSP1-induced migration, suggesting that, whereas hep I is not

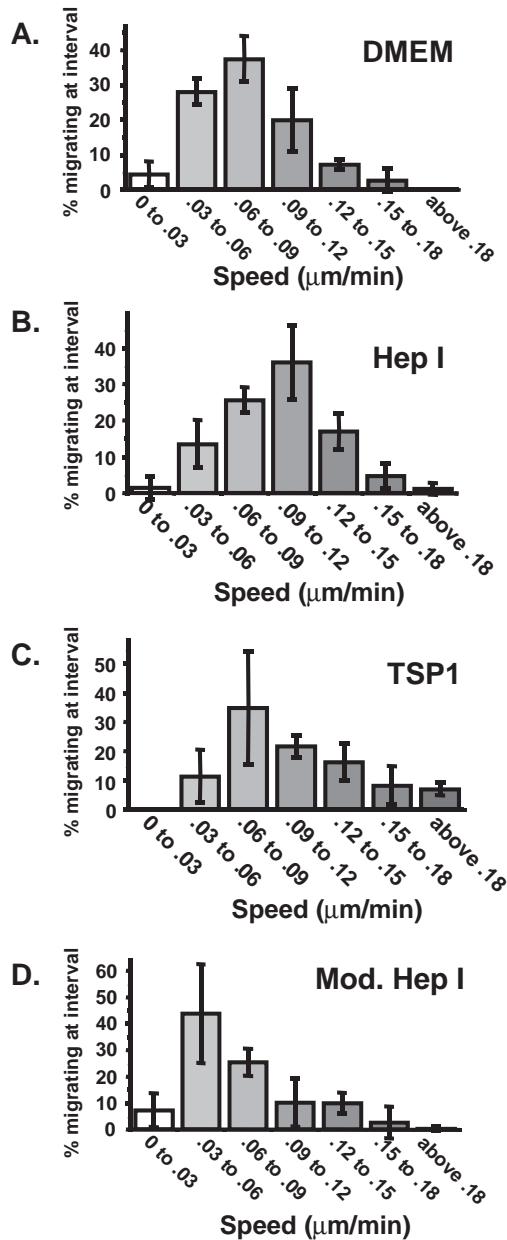


Fig. 4. Histogram for endothelial migration speeds following TSP1/hep I treatment. BAE cells were plated at low density under serum-free conditions onto glass coverslips coated with vitronectin and fibronectin. Cells were allowed to attach for 3 hours and loaded onto the Dunn Chamber in serum-free media (A), or media containing either hep I (100 nM) (B), TSP1 (7.8 nM) (C) or modified hep I (100 nM) (D). The chamber was sealed, and time-lapse video was taken of the cells over a 7-hour time span. Migration of individual cells was tracked using Metamorph software and migration speed was determined. Cells were separated into consecutive speed intervals and the percent of cells migrating at each interval was determined. At least 75 cells were analyzed per condition. $n=3-4$.

the only pro-migratory signal elicited by TSP1, signaling through the hep I sequence is required for maximal TSP1-induced fibroblast migration.

MEFs deficient for LRP, the second component of the

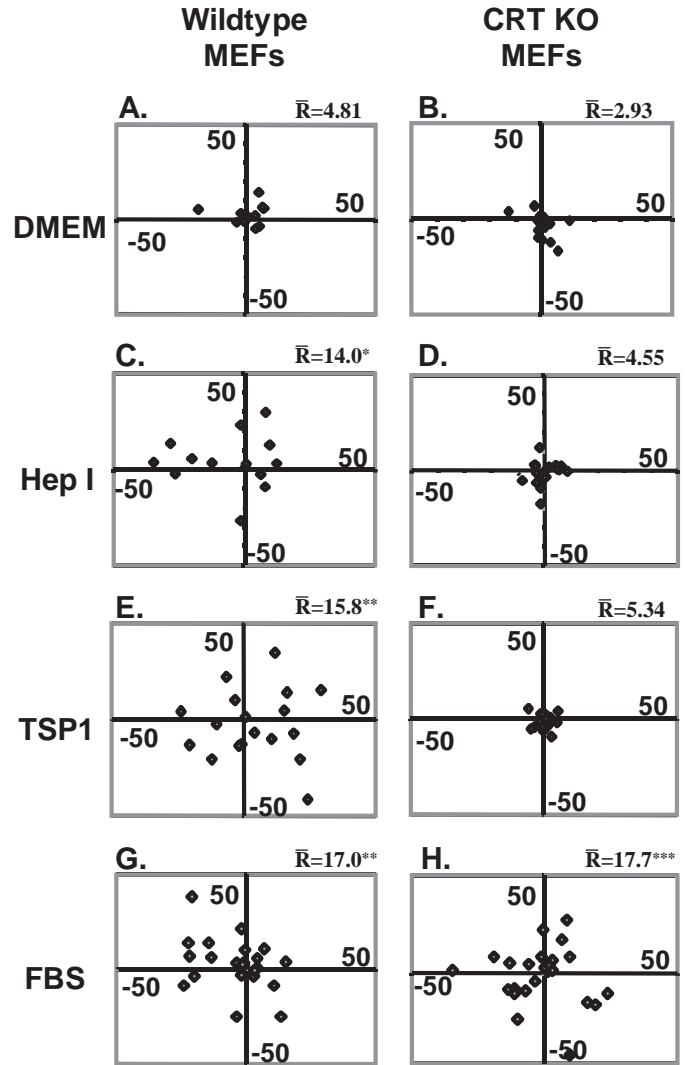


Fig. 5. CRT-knockout fibroblasts do not migrate in response to TSP1/hep I. Wild-type and CRT-knockout (KO) MEFs were plated at low density under serum-free conditions onto glass coverslips coated with vitronectin and fibronectin. Cells were allowed to attach for 3 hours and loaded onto the Dunn Chamber in serum-free media (A,B), with or without 100 nM hep I (C,D), 7.8 nM TSP1 (E,F) or 0.1% FBS (G,H). The chamber was sealed, and time-lapse video was taken of the cells over a 7-hour time span. Migration of individual cells was tracked using Metamorph software and individual tracks were analyzed for distance, displacement, directionality and orientation. Final positions of the cells are plotted with the initial point being the origin. Results are representative of at least three separate experiments. Average displacement (\bar{R}) is given for each treatment. $*P<0.05$, $**P<0.01$, $***P<0.001$.

TSP1/hep I receptor complex, demonstrated a similar inability to respond to TSP1/hep I signaling (Fig. 7A-D). However, unlike CRT-knockout MEFs, LRP-knockout MEFs do not show significant migration in response to 0.1% FBS treatment, suggesting a general migratory defect in these cells (Movie 3, available at jcs.biologists.org/supplemental). Consistent with this, LRP-knockout cells did not migrate in response to treatment with bFGF or tenascin C (data not shown). To demonstrate that LRP is actually involved in TSP1/hep I-

Fig. 6. Differences in fibroblast migration to TSP1 and hep I. (A) Wild-type and CRT-knockout (KO) MEF migration in response to serum-free media, with or without hep I (100 nM), TSP1 (7.8 nM) or FBS (0.1%), was assessed as previously described. Average cell migration speed under each condition was determined by dividing total migration distance by the total time of the assay. At least 75 cells were analyzed for each condition. $n=3-4$, * $P<0.05$, ** $P<0.01$. (B) Percent of cells migrating under each condition was determined by dividing the number of cells migrating at least 0.06 microns/minute by the total number of cells. At least 75 cells were analyzed for each condition. $n=3-4$, * $P<0.05$, ** $P<0.01$. (C) Total cellular displacement was determined as the difference between final cell position and initial cell position. At least 75 cells were analyzed for each condition. $n=3-4$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$. (D) Directionality of cell migration was determined by dividing the total cellular displacement by the total distance of migration. Under this method, a purely directional response will show a ratio of 1, whereas increasingly random migration responses will show ratios approaching zero. At least 75 cells were analyzed for each condition. $n=3-4$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

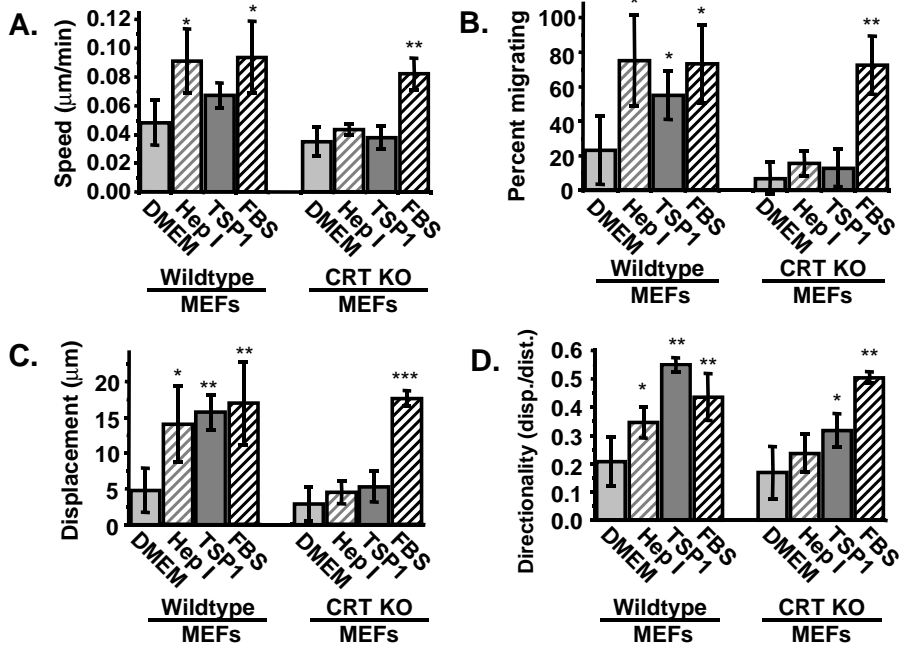
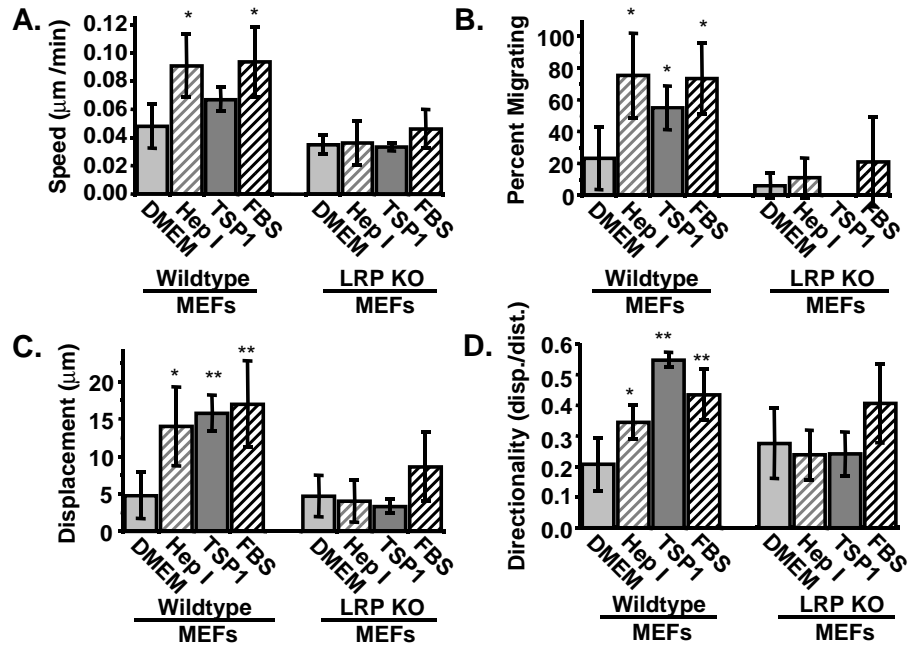


Fig. 7. LRP-knockout fibroblasts are migration deficient. (A) Wild-type and LRP-knockout (KO) MEF migration in response to serum-free media, with or without hep I (100 nM), TSP1 (7.8 nM) or FBS (0.1%), was assessed as previously described. Average cell migration speed under each condition was determined by dividing total migration distance by the total time of the assay. At least 75 cells were analyzed for each condition. $n=3-4$, * $P<0.05$, ** $P<0.01$. (B) Percent of cells migrating under each condition was determined by dividing the number of cells migrating at least 0.06 microns/minute by the total number of cells. At least 75 cells were analyzed for each condition. $n=3-4$, * $P<0.05$, ** $P<0.01$. (C) Total cellular displacement was determined as the difference between final cell position and initial cell position. At least 75 cells were analyzed for each condition. $n=3-4$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$. (D) Directionality of cell migration was determined by dividing the total cellular displacement by the total distance of migration. Under this method, a purely directional response will show a ratio of 1, whereas increasingly random migration responses will show ratios approaching zero. At least 75 cells were analyzed for each condition. $n=3-4$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.



induced migration, endothelial cells were pre-incubated with receptor-associated protein (RAP) (50 nM), which is an endogenous LRP inhibitor, and stimulated with TSP1/hep I. This concentration of RAP is sufficient to inhibit focal adhesion disassembly and intracellular signaling in response to TSP1/hep I treatment (Orr et al., 2003). Resulting migratory responses are shown by scatter plots of final cell position, which is an indication of total cellular displacement (R).

Whereas RAP alone did not affect endothelial cell migration (Fig. 8A,B), RAP pre-treatment significantly inhibited the ability of endothelial cells to respond to TSP1/hep I-induced chemokinesis (Fig. 8C-F). RAP has no significant effect on FBS-induced endothelial cell migration (Fig. 8G,H). Thus, TSP1/hep I stimulates endothelial cell and fibroblast chemokinesis through the previously described CRT- and LRP-dependent focal adhesion-labilizing pathway.

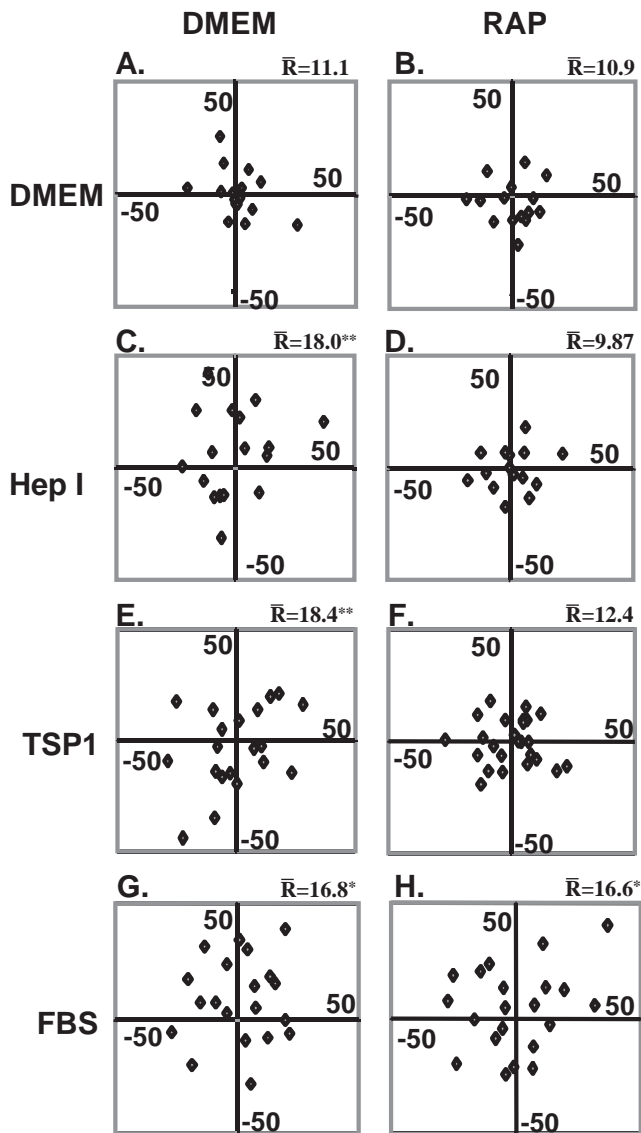


Fig. 8. RAP pretreatment blocks TSP1/hep I-induced endothelial cell migration. BAE cells were plated at low density under serum-free conditions onto glass coverslips coated with vitronectin and fibronectin. Cells were allowed to attach for 3 hours. Some coverslips were pretreated for 30 minutes with the LRP inhibitor receptor-associated protein (RAP) at 50 nM. Coverslips were loaded onto the Dunn Chamber in serum-free media (A,C,E,G) or serum-free media containing RAP (B,D,F,H), as well as 100 nM hep I (C,D), 7.8 nM TSP1 (E,F) or 0.1% FBS (G,H). The chamber was sealed, and time-lapse video was taken of the cells over a 7-hour time span. Migration of individual cells was tracked using Metamorph software and individual tracks were analyzed for distance, displacement, directionality and orientation. Final positions of the cells are plotted with the initial point being the origin. Results are representative of at least three separate experiments. Average displacement (\bar{R}) is given for each treatment. * $P < 0.05$, ** $P < 0.01$.

Chemokinetic hep I treatment alters endothelial cell migration toward aFGF and bFGF

Cells encounter numerous pro-migratory and anti-migratory stimuli, each with their own unique attributes, which the cell integrates into a specific migratory response. Cell-ECM

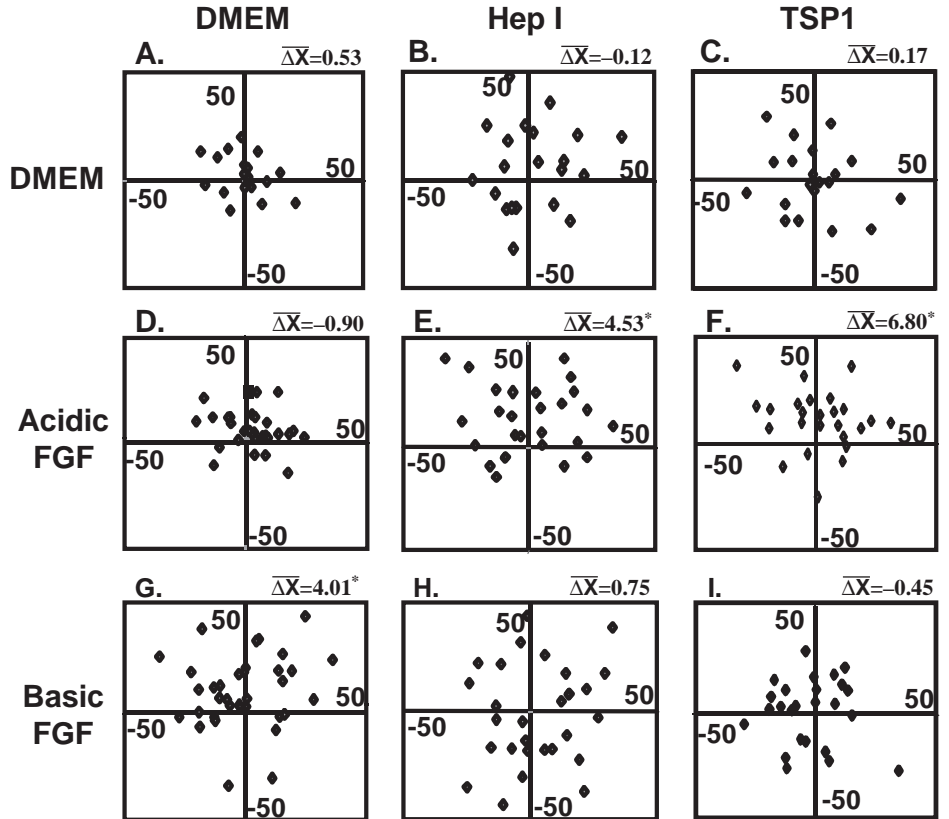
adhesions, particularly focal adhesions, can act as sites of integration between growth factor-derived and matrix-derived signals, but the effect of altering these structures on the integration of these signals has not been fully developed (Hauck et al., 2002; Eliceiri, 2001). Thus, we sought to determine the effect of TSP1/hep I-induced chemokinesis, which involves modulation of focal adhesion structure, on chemotaxis towards separate growth factors, such as aFGF and bFGF. Cells were loaded onto the Dunn chamber using either serum-free media or media containing hep I peptide (100 nM) or TSP1 (7.8 nM), with the outer well also containing either aFGF (67 pM) or bFGF (61 pM), forming a chemotactic gradient. This allowed us to observe aFGF- and bFGF-induced chemotaxis in the presence or absence of focal adhesion-labilizing concentrations of hep I peptide and TSP1. Treatment with an aFGF gradient induced only a slight chemotactic response in BAE cells (Fig. 9A,D; Movies 1 and 4, available at jcs.biologists.org/supplemental), whereas bFGF stimulated a potent chemotactic response (Fig. 9A,G; Movies 1 and 5, available at jcs.biologists.org/supplemental). When added in the presence of hep I or TSP1, aFGF displayed enhanced chemotactic properties (Fig. 9B,E and Fig. 9C,F; Movies 2 and 6, available at jcs.biologists.org/supplemental), whereas bFGF showed a reduced ability to orient BAE cell migration (Fig. 9B,H and Fig. 9C,I; Movies 2 and 7, available at jcs.biologists.org/supplemental). Hep I neither enhanced nor inhibited the ability of 0.1% FBS to stimulate endothelial cell chemotaxis, probably reflecting differing effects on the multiple growth factors that are present in serum (data not shown). These data suggest that the ability of cells to respond to specific growth factors might depend on the adhesive state of the cell.

Discussion

TSP1 expression during pathological processes, such as wound healing, atherosclerotic plaque development, angiogenesis and tumor metastasis, is well established (Adams, 2001; Bornstein, 2001). However, the ability of TSP1 to signal through multiple domains and cell-surface receptors, eliciting a wide range of biological effects, has hindered attempts to determine the precise role of TSP1 in the development and progression of these disease processes (Adams, 2001; Bornstein, 2001). Although TSP1 has long been known to stimulate focal adhesion disassembly in firmly adherent cells, resulting in a transition to an intermediate adhesive state, the role of this signaling event in TSP1 biology has not been characterized. We now report that signaling by the hep I sequence in TSP1 through cell-surface CRT and LRP results in the modulation of migratory phenotype in firmly adherent endothelial cells and fibroblasts. This phenotypic change is characterized by increased chemotaxis towards a soluble TSP1/hep I gradient, random migration in the presence of focal adhesion-labilizing TSP1/hep I concentrations, and altered chemotaxis to aFGF and bFGF.

TSP1 differentially affects migration of certain cell types in response to the changing context of the ECM. Several studies have been performed describing both a pro-migratory and an anti-migratory effect of TSP1 on endothelial cell migration. TSP1 was the first endogenous anti-angiogenic molecule discovered, and the ability of TSP1 to inhibit angiogenesis results from the pro-apoptotic and anti-migratory effect of the CSVTCG sequence in the type 1 repeats of TSP1 signaling

Fig. 9. Hep I modulates aFGF/bFGF-induced endothelial cell chemotaxis. BAE cells were plated at low density under serum-free conditions onto glass coverslips coated with vitronectin and fibronectin. Cells were allowed to attach for 3 hours. Some coverslips were pretreated for 30 minutes with 100 nM hep I or 7.8 nM TSP1. Coverslips were loaded onto the Dunn Chamber in serum-free media (A,D,G) or serum-free media containing either 100 nM hep I (B,E,H) or 7.8 nM TSP1 (C,F,I). Media was removed from the outer well and corresponding media containing aFGF (67 pM) (D,E,F; Movies 4 and 6, available at jcs.biologists.org/supplemental) or bFGF (61 pM) (G,H,I; Movies 5 and 7, available at jcs.biologists.org/supplemental) was then loaded into the outer well establishing a chemical gradient. The chamber was sealed, and time-lapse video was taken of the cells over a 7-hour time span. Migration of individual cells was tracked using Metamorph software and individual tracks were analyzed for distance, displacement, directionality and orientation. Final positions of the cells are plotted with the initial point being the origin and the top of the graph representing the outer well. Results are representative of at least six separate experiments. Average displacement towards the outer well ($\overline{\Delta X}$) is given for each treatment. * $P < 0.05$, ** $P < 0.01$.



through CD36 on endothelial cells (Good et al., 1990; Dawson et al., 1997). However, endothelial cells show differential expression of CD36 based on their location, with no CD36 expressed on large vessel endothelial cells (Febbraio et al., 2001). Since angiogenesis occurs mostly through capillary endothelial cells, TSP1 is likely to be primarily anti-angiogenic. However, TSP1 may stimulate large vessel endothelial cell migration, enhancing their ability to respond to vascular wounding. Furthermore, platelet-derived histidine-rich glycoprotein binds TSP1 and inhibits TSP1-induced CD36 activation (Simantov et al., 2001). Under these conditions, cellular responses to other domains of TSP1, such as the HBD, might be selectively enhanced. Consistent with this, the HBD of TSP1 stimulates angiogenesis and upregulates the pro-angiogenic proteins matrix metalloproteinase (MMP)-9 and tissue inhibitor of metalloproteinase (TIMP)-1 (Ferrari do Outeiro-Bernstein et al., 2002; Qian et al., 1997; Taraboletti et al., 2000). HBD-induced angiogenesis might involve hep I signaling, since both TSP1-induced focal adhesion disassembly and HBD-induced angiogenesis are inhibited by heparin (Murphy-Ullrich et al., 1993; Ferrari do Outeiro-Bernstein et al., 2002). Signaling through the HBD might be enhanced by the release of this domain from full-length TSP1 through the actions of several endogenous proteases, thus separating the pro- and anti-angiogenic signals of TSP1. In addition to hep I signaling, TSP1 might also enhance endothelial cell migration through interactions with the $\alpha_3\beta_1$ integrin and syndecans (Lakshmi et al., 2000; Ferrari do Outeiro-Bernstein et al., 2002). The ability of $\alpha_3\beta_1$ integrin,

heparan sulfate proteoglycans (HSPGs) and CRT/LRP to mediate TSP1-induced cell migration might reflect synergism in TSP1-derived pro-migratory signals, multiple sites of regulation in the migration process, or environment-specific pro-migratory stimuli.

TSP1/hep I stimulates focal adhesion disassembly and cell migration through the CRT-LRP receptor co-complex. Whereas basal cell migration is unaffected by CRT inhibition, LRP-knockout cells demonstrate a severe migration deficiency. In addition to being non-responsive to TSP1/hep I-induced cell migration, LRP-knockout cells do not migrate in response to treatment with FBS, tenascin C or bFGF. Since these cells lose focal adhesions but do not migrate in response to tenascin C treatment, the migration deficiency in LRP-knockout MEFs is not due to a defect in focal adhesion turnover. This suggests that additional signals might be necessary for migration. LRP-knockout cells exhibit reduced cell spreading compared with wild-type cells, and time-lapse video microscopy demonstrates that LRP-knockout MEFs do not extend large, stable lamellipodia (Movie 3, available at jcs.biologists.org/supplemental; A. W. Orr, D. Kucik and J. E. Murphy-Ullrich, unpublished). In apparent contradiction to this, recent work (Ma et al., 2002) indicates that LRP-knockout fibroblasts display increased activation of Rac, a small GTPase known to play a pivotal role in spreading and lamellipodia formation. However, in these studies, experiments were performed under high serum conditions: in the absence of LRP, the stability of Rac-activating growth factors might be enhanced because of a lack of LRP-mediated endocytosis and clearance of activating factors.

A role for LRP in determining basal cell migration rate has not previously been described although, under high serum conditions, LRP might modulate growth factor- and protease-dependent cell migration (Ma et al., 2002). LRP-dependent endocytosis inhibits migration in response to urokinase-type plasminogen activator (uPA) binding to its receptor (uPAR) (Weaver et al., 1997). However, LRP mediates migration in response to complexes of plasminogen activator inhibitor-1 (PAI-1) with uPAR-uPA, as well as platelet-derived growth factor (PDGF)-induced cell migration through LRP transphosphorylation (Chazaud et al., 2000; Chazaud et al., 2002; Loukinova et al., 2002). LRP localizes to the leading edge of migrating breast cancer cells, and LRP expression correlates with breast cancer cell invasiveness (Chazaud et al., 2002; Li et al., 1998-1999), suggesting that localized LRP signaling within the lamellipodia might regulate normal protrusive activity and cell migration. However, preincubation of endothelial cells with RAP, an endogenous LRP inhibitor, does not reduce basal cell migration, indicating that ligand binding may not be the primary function of LRP in determining basal cell migration rate.

aFGF and bFGF have long been associated with endothelial cell migration, vascular development and angiogenesis (Joseph-Silverstein and Rifkin, 1987; Slavin, 1995). These heparin-binding growth factors, although similar in structure and function, display distinct differences. aFGF alone does not significantly affect BAE cell migration and requires co-stimulation with heparin to mediate maximal endothelial cell responses (Joseph-Silverstein and Rifkin, 1987; Slavin, 1995), whereas, bFGF does not require complementary signals to stimulate endothelial cell migration and proliferation maximally. Our studies show that hep I treatment enhances aFGF-induced chemotaxis but inhibits chemotaxis to bFGF. It has previously been reported that TSP1 inhibits bFGF-induced endothelial cell migration and angiogenesis (Vogel et al., 1993), although this occurs through heparin-binding sequences in the type 1 repeats of TSP1 and not the amino-terminal HBD. Since both TSP1 and bFGF bind HSPGs, the ability of TSP1 to inhibit bFGF-induced responses is suggested to occur through competitive inhibition for binding to HSPGs (Vogel et al., 1993). However, whereas both TSP1 and bFGF bind the HSPG perlecan on endothelial cells, the sites mediating these interactions are distinct (Feitsma et al., 2000). In addition, hep I-induced focal adhesion disassembly is insensitive to heparitinase treatment, indicating that hep I does not bind endothelial cell HSPGs and thus does not inhibit bFGF signaling through competition for endogenous HSPGs (Murphy-Ullrich et al., 1993).

Cells integrate environmental signals, such as growth factors and ECM proteins, to determine context-specific responses (Hauck et al., 2002; Eliceiri, 2001). Although integrin-mediated adhesion regulates the ability of growth factors to induce certain cellular responses, little is known about the consequence of altering adhesion complexes on growth factor signaling. Focal adhesion disassembly can potentially alter adhesion-derived signaling and the resulting growth factor responses. Stimulation with aFGF results in endothelial cell focal adhesion disassembly, whereas bFGF does not alter focal adhesion dynamics, suggesting that bFGF targets other migratory processes (Ding et al., 2000; Lee and Gotlieb, 2002) (A. W. Orr, M. A. Pallero and J. E. Murphy-Ullrich,

unpublished). bFGF stimulates endothelial cell chemotaxis through FGF receptor-1 (FGFR-1), which localizes to focal adhesions, and activation of ERK within focal adhesions is required for bFGF-induced endothelial cell migration (Shono et al., 2001; Tanghetti et al., 2002). Alterations in focal adhesion structure might alter receptor localization or activation of focal adhesion-derived signaling pathways required for bFGF-induced chemotaxis. Although both aFGF and bFGF stimulate chemotaxis through FGFR-1, no requirement for receptor localization to focal adhesions has been described for aFGF function (Slavin, 1995). Furthermore, since aFGF induces a similar loss of focal adhesion structure, TSP1-induced intermediate adhesive signals probably affect aFGF and bFGF signaling differently.

The work presented herein demonstrates that TSP1 induces a phenotypic switch in endothelial cell migration, both enhancing migration on its own and specifically modulating endothelial cell responses to the FGF family of growth factors. In addition to endothelial cells, TSP1 stimulates focal adhesion disassembly in every adherent cell type tested thus far, including both fibroblasts and smooth muscle cells, suggesting TSP1-mediated focal adhesion disassembly might have a more universal effect on localized areas of active cell migration. Furthermore, this work suggests a role for LRP in the regulation of basal cell motility. Future work will further characterize the role of this migratory response in various pathological states and explore the potential changes in proliferation, apoptosis and gene expression induced in response to this TSP1-derived signal.

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