

Epidermal growth factor alters fibroblast migration speed and directional persistence reciprocally and in a matrix-dependent manner

Margaret F. Ware¹, Alan Wells² and Douglas A. Lauffenburger¹

¹Center for Biomedical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

²Department of Pathology, University of Alabama-Birmingham, and Birmingham VAMC, Birmingham, AL 35294, USA

*Author for correspondence (e-mail: lauffen@mit.edu)

Accepted 9 June; published on WWW 30 July 1998

SUMMARY

Growth factors stimulate sustained cell migration as well as inducing select acute motility-related events such as membrane ruffling and disruption of focal adhesions. However, an in-depth understanding of the characteristics of sustained migration that are regulated by growth factor signals is lacking: how the biochemical signals are related to physical processes underlying locomotion, and how these events are coordinately influenced by interplay between growth factor and matrix substratum signals. To address these issues, we studied sustained migration of NR6 fibroblasts on a complex human matrix substratum, Amgel, comparing effects of epidermal growth factor (EGF) treatment across a range of Amgel levels. In the absence of EGF, cell migration speed and directional persistence are relatively independent of Amgel level, whereas in the presence of EGF speed is increased at intermediate Amgel levels but not at low and high Amgel levels while directional persistence is decreased at intermediate but not at low and high Amgel levels. The net effect of EGF is to increase the frequency of changes in the cell direction, and at the

same time to slightly increase the path-length and thereby greatly enhance random dispersion of cells. Despite increasing migration speed during long-term sustained migration EGF treatment does not lead to significantly increased absolute rates of membrane extension in contrast to its well-known elicitation of membrane ruffling in the short term. However, EGF treatment does decrease cell spread area, yielding an apparent enhancement of specific membrane extension rate, i.e. normalized to cell spread area. Cell movement speed and directional persistence are thus, respectively, directly related and indirectly related to the increase in specific membrane extension rate (alternatively, the decrease in cell spread area) induced by EGF treatment during sustained migration. These results indicate that growth factor and matrix substrata coordinately regulate sustained cell migration through combined governance of underlying physical processes.

Key words: Motility, Growth factor, Extracellular matrix

INTRODUCTION

Cell migration plays an important role in many physiological and pathological processes. Tumor progression to invasive and metastatic states depends, at least in part, on active cell motility of invading tumor cells (Chicoine and Silbergeld, 1995; Turner et al., 1996, 1997; Radinsky et al., 1995; Xie et al., 1995) and of endothelial cells during tumor neovascularization (Chen et al., 1997; Thommen et al., 1997; Wijelath et al., 1997). Evidence is accumulating that growth factor induced migration, rather than constitutive haptokinetic motility, is crucial for both the tumor cell invasion and the endothelial cell ingrowth involved. EGF receptor (EGFR), the receptor most commonly associated with human tumors (Aaronson, 1991), has been implicated in both processes (Chakrabarty et al., 1995; Engebraaten et al., 1993; Li et al., 1993; Nelson et al., 1995; Radinsky et al., 1995; Turner et al., 1996; Ushiro et al., 1996). Understanding EGFR-mediated regulation of cell migration may thus provide novel approaches to controlling tumor spread.

Cell migration paths are dictated by both the rate of locomotion (migration speed) and the time period over which the cell front continues in roughly the same direction (directional persistence) (Gail and Boone, 1970; Dunn, 1983). These defining parameters are, in turn, governed by specific biophysical events such as forward protrusion of lamellipodia, formation of new attachments to substratum, generation of contractile forces to translocate the cell body, and rear detachment from the substratum (Lauffenburger and Horwitz, 1996). Alterations in a spectrum of processes, including cytoskeletal organization, integrin-mediated cell/substratum linkages, and intracellular force generation, can be plausibly envisioned as rate-limiting steps augmented by EGFR signaling. At present, however, it is not known whether EGF-enhanced cell movement occurs by means of effects on linear translocation speed or on directional persistence, or both. One may gain new insights into the underlying biochemical and biophysical mechanisms by determining how EGFR activation alters these parameters.

EGFR signaling stimulates cell motility in adherent cells such as endothelial cells, fibroblasts and epithelial cells (Ando and Jensen, 1993; Cha et al., 1996; Chen et al., 1994a; Maldonado and Furcht, 1995; Matthay et al., 1993). Progress has been made in identifying motility-related intracellular events induced by EGF. Required downstream signaling pathways include one via PLC γ causing mobilization and activation of actin-modifying proteins such as gelsolin leading to reorganization of the actin cytoskeleton (Chen et al., 1994b, 1996) and another via MAP kinase leading to focal adhesion disassembly and decreased adhesiveness (Xie et al., 1998); both of these pathways are activated by EGF for extended time periods (Chen et al., 1994b; H. Shiraha and A. Wells, unpublished observations). Acute activation of additional pathways, involving rho and rac, result in increased membrane ruffling and filipodial formation, respectively (Ridley and Hall, 1992; Ridley et al., 1992), though the link to enhancement of sustained cell migration is uncertain at present. The same pathways have been shown to be required for induction of cell motility by other growth factors (Bornfeldt et al., 1994; Kundra et al., 1994; Ridley et al., 1995). A key unanswered question is what are the specific physical processes by which EGFR-mediated signaling leads to enhanced cell migration.

The focus of our work here is to determine how EGF affects fibroblast migratory characteristics, specifically speed and persistence time. Previous studies usually investigate the overall dispersion of cell populations (Cha et al., 1996; Chen et al., 1994a,b; Klemke et al., 1997) While these approaches can yield data on overall cell movement responses, they do not provide detailed information about characteristics of cell movement. Therefore, we used individual cell tracking to determine the parameters of speed and directional persistence time, along with the fraction of cells locomoting. As substratum ligand density and cell adhesion affect cell motility (Duband et al., 1991; Goodman et al., 1989; DiMilla et al., 1993; Palecek et al., 1997), we determined EGF-induced motility over a range of substratum protein concentrations. As a model system, we utilized NR6 cells, mouse 3T3 derivatives lacking endogenous EGFR, expressing wild-type EGFR (Chen et al., 1994a,b, 1996). We found that EGFR activation increased cell movement speed while reducing directional persistence, yielding greater cell dispersion (or 'scattering') with more erratic cell paths. This induced motility was affected by substratum density in a biphasic manner similarly to haptokinetic migration (DiMilla et al., 1993; Palecek et al., 1997). In an initial examination of the underlying biophysical bases of EGFR-mediated motility, we found that EGF elicits an increase in lamellipodial extension relative to the cell spread area. These findings suggest a model in which growth factors induce motility by stimulating membrane extensions in a non-directional manner.

MATERIALS AND METHODS

Materials

MEM α , sodium pyruvate, non-essential amino acids, penicillin/streptomycin, L-glutamine, geneticin/G418 sulfate, trypsin EDTA, DPBS, fetal bovine serum, and recombinant hEGF were all

obtained from Gibco BRL (Grand Island, NY). Dialyzed fetal bovine serum (dFBS) was from Sigma (St Louis, MO). Tissue culture flasks and 35 mm suspension culture dishes were obtained from Corning (Cambridge, MA).

Cell lines and culture

The NR6 cells expressing EGFR constructs were generated as described previously (Chen et al., 1994a). Briefly, the full length EGFR cDNA was derived from human placental isolate (Welsh et al., 1991). This construct was stably expressed in NR6 cells, 3T3 derivatives which are devoid of endogenous EGFR (Pruss and Herschman, 1977), using retroviral-mediated transduction (Wells and Bishop, 1988). This subline, WT NR6, responds to EGF in a manner indistinguishable from parental 3T3 cells which express endogenous EGFR (Chen et al., 1994a,b, 1996; Wells et al., 1990; Welsh et al., 1991). The cell line was routinely passaged in MEM α supplemented with 7.5% FBS and 350 μ g/ml G418. Pre-confluent 75 cm² tissue culture flasks were split 1:5 every two days. Cells were frozen in MEM α supplemented with 10% DMSO and 30% FBS.

Substratum preparation

Non-tissue culture suspension dishes were coated with varying levels of Amgel. Amgel is a biologically-active extracellular matrix derived from human placental amniotic membranes containing type IV collagen, laminin, entactin, tenascin, and heparan sulfate proteoglycan; it does not contain detectable levels of EGF, TGF α , TGF β or PDGF (Siegal et al., 1993; Xie et al., 1995), all of which could introduce confounding signals in our assays. Various concentrations of Amgel in PBS (0.014 to 48 μ g/ml) were incubated in 35 mm dishes at room temperature for 1 hour and 15 minutes. The solution was aspirated, and the plates were blocked for 1 hour with 0.1% BSA. The plates were then washed thrice with PBS and stored in PBS at 4°C for up to 10 days. The Amgel concentrations mentioned throughout refer to the coating concentration, as the composition of the substratum during the migration assays may be altered due to nonspecifically adsorbed proteins.

Cell migration assays

Experiments were conducted in 35 mm non-tissue culture plates that had been coated with Amgel. Approximately 4,000 cells were seeded in normal growth medium and allowed to attach overnight. The low cell density, covering approximately 0.5% of the surface area, was chosen to minimize any cell/cell interactions over the course of the experiment. The cells were then switched to serum-free medium for 24 hours to induce quiescence. For control experiments cells were then switched to MEM α medium without bicarbonate, but with 20 mM Hepes (pH 7.4) for the air atmosphere, 0.1% BSA, (M/H/B) and 1% dFBS. For experiments involving growth factor, a saturating concentration of EGF (25 nM) was also added. Due to the high EGF concentration and low cell density, depletion of growth factor over the course of the experiment does not occur (Reddy et al., 1994). Furthermore, under these conditions, both PLC and MAP kinase demonstrate activation 5-24 hours after addition of EGF (Chen et al., 1994b; H. Shiraha and A. Wells, unpublished observations). Cells were incubated for 8 hours in a warmed, humidified, non-CO₂ environment.

The 8 hour incubation is due to the induction effects caused by the addition of growth factor. The average mean squared displacements of all the cells for subsequent 30 minute time intervals were examined. The data indicate (data not shown) that migration increases over a period of 6 to 8 hours following addition of growth factor. For this reason we pre-incubated the cells for 8 hours in EGF before we began capturing centroids for analysis.

Motility parameters were determined by tracking single cells for a minimum of 10 hours and a maximum of 20. Cells were placed into a motorized stage (LUDL electronics, Hawthorne, NY) and were

observed using a Zeiss Axiovert 35 inverted phase contrast microscope. A $\times 10$ objective and $\times 10$ eyepiece were used for a final magnification of $\times 100$. A 37°C environment was maintained by circulating warm water through the stage. To prevent evaporation of the medium over the course of the experiment, mineral oil was overlaid on the medium to provide a sealed environment that still allows gas exchange. Cell centroid data were acquired using a nuLogic stage controller connected to a power Macintosh running Labview software (National Instruments, Bloomfield, CT). In a given experiment, centroids from an average of 50 cells were obtained every 15 minutes from 12 different fields. Visual data were acquired using a Sony CCD-IRIS camera and Panasonic AG-6040 time-lapse VCR. Centroid positions were transferred to a spreadsheet program and mean squared displacements ($\langle d^2 \rangle$) over time were calculated using the method of non-overlapping intervals (Dickinson and Tranquillo, 1993). The speed, S , was determined by the 15-minute mean-squared displacement, and directional persistence time, P , were obtained by fitting these to the persistent random walk equation (Dunn, 1983; Othmer et al., 1988):

$$\langle d^2(t) \rangle = 2S^2P[t - P(1 - e^{-t/P})],$$

where t is the time interval, using a non-linear least squares regression analysis. Conceptually, speed can be considered as the total path length, however tortuous, over time, and persistence the length of time a cell remains moving without changing direction by more than 60° . A representative cell path and curve fit are shown in Fig. 1. Once the parameters were determined for individual cells, cell population histograms were generated for each condition. By examining the full histogram of the data, more information is gathered than if the mean squared displacements were first averaged and then fit to the equation. The mean and 95% standard error were calculated for each histogram and reported as the speed and persistence times for each of the conditions. 95% standard error of the mean provides an estimate of the accuracy of our mean, and we can be $(1-\alpha)100\%$ confident that the error will be less than

$$(stdev)(timv)/\sqrt{n-1},$$

where $timv = f(\alpha, n-1)$ is the inverse of Student's t distribution, $stdev$ is the standard deviation, and n is the sample size.

Membrane extension determination

Membrane extension experiments were performed as described for the migration experiments with minor modifications. Cells were observed under a $\times 32$ objective in order to observe any membrane movement. Individual cell images were captured using Scion Image from video

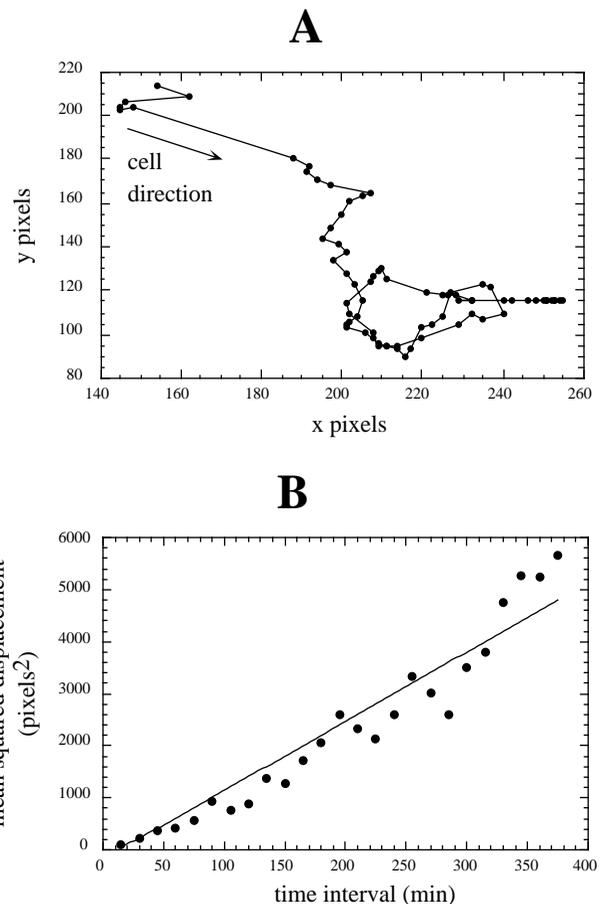


Fig. 1. Determination of experimental parameters. Typical cell path (A) and curve fit (B) for cells incubated at $0.048 \mu\text{g/ml}$ Amgel and 25 nM EGF. The markers in A represent the cell centroid position at 15 minute intervals.

tapes with time signatures. Each cell was observed for 60 to 120 minutes at 15 minute intervals. Individual cells were outlined and then overlaid one at a time (Fig. 2) to determine two dimensional cell protrusion and retraction areas.

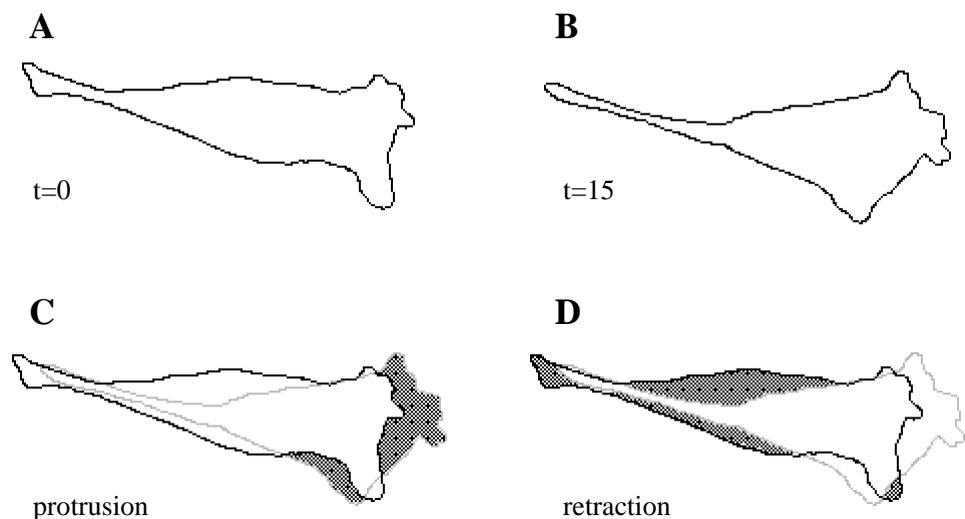


Fig. 2. Schematic for calculation of membrane extension. Each cell is outlined at 15 minute intervals (A,B). The outlines are then overlaid, with the later time represented in gray. Protrusion area (C) and retraction area (D) are filled in and the resultant area is calculated.

RESULTS

EGF increases individual cell locomotion

We first wanted to confirm a stimulatory effect of EGF on cell locomotion (Chen et al., 1994a; Wilson et al., 1994; Matthay et al., 1993; Maldonado and Furcht, 1995). Fig. 3 shows the effect of 25 nM EGF on behavior of NR6 cells expressing the WT EGFR at an Amgel coating concentration of 0.048 $\mu\text{g/ml}$. As can be seen in this figure, the cells in the presence of EGF show a highly asymmetric morphology, generally indicative of locomotion, whereas the untreated cells remain more symmetrically spread. EGF treatment causes cells to move 1 or 2 cell lengths in this one hour time period, while untreated cells remain almost stationary despite exhibiting noticeable membrane extension activity.

One simple way to quantify the EGF effect is by the fraction of cells that are stimulated to locomote. For instance, we can define non-motile cells as those that never move even one cell length ($<50\ \mu\text{m}$) in a single hour over the 20 hour course of an experiment; motile cells are defined to be those moving at least one cell length ($>70\ \mu\text{m}$) in one hour at some point during the experiment. At 0.48 $\mu\text{g/ml}$ Amgel coating concentration, for

example, in the absence of EGF 96% of the cells are non-motile while 4% are motile, whereas in the presence of EGF 80% are motile and 20% are non-motile. However, we believe that information more suitable for elucidating mechanistic underpinnings arises from more detailed measurements of migration speed and persistence, as reported below.

EGF-induced motility is constrained by substratum density

Our previous studies have demonstrated that haptokinetic motility is limited in a biphasic manner by adhesiveness of cells to the substratum (DiMilla et al., 1993; Palecek et al., 1997). In short, if a surface is either too 'slippery' or too 'sticky', cells will not locomote. Therefore, we determined if growth factor-induced motility is similarly constrained. We examined EGF motogenic effects over a range of concentrations of the substratum Amgel, a biologically-active extracellular matrix isolated from human amniotic membranes (Siegal et al., 1993). We utilized this complex ECM instead of individual components as a closer mirror of the *in vivo* situation.

Fig. 4 shows the difference in cell tracks as a result of the substratum concentration. Five random, individual cell tracks

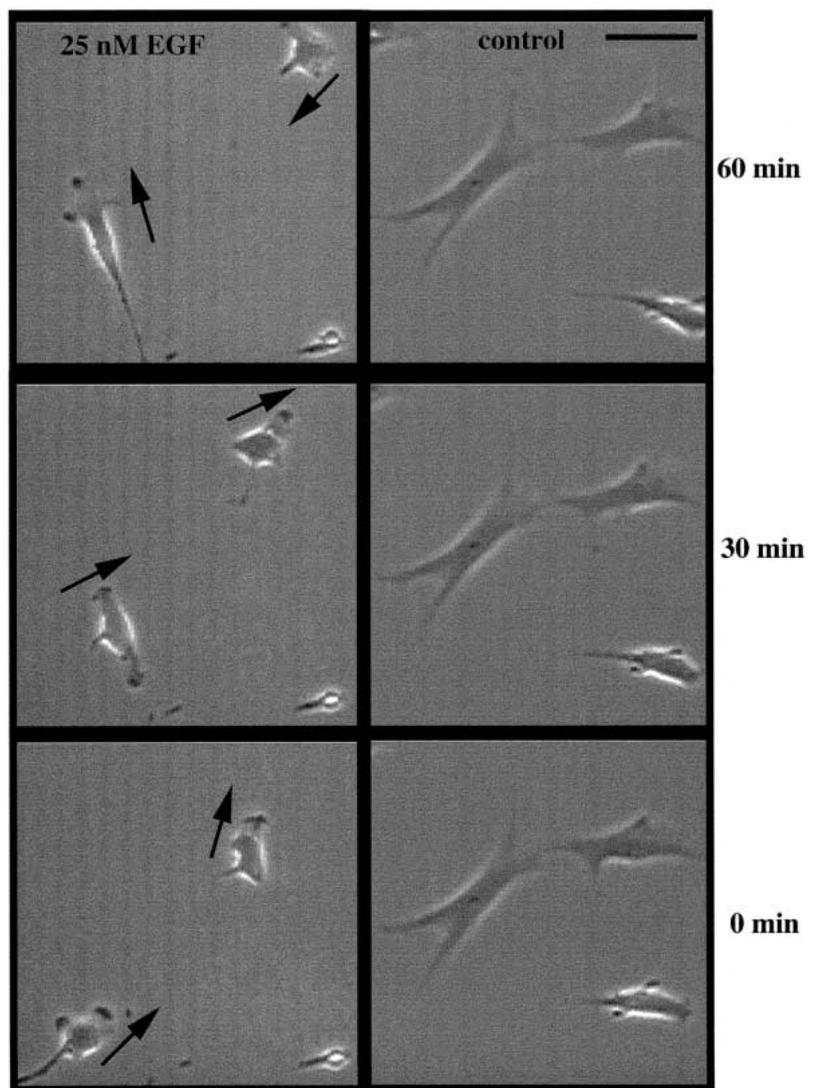


Fig. 3. Morphological changes due to EGF. NR6 cells display a marked change in morphology upon treatment with 25 nM EGF. The right three panels are cells without EGF and the left three panels are cells in 25 nM EGF after at least 8 hours of incubation. Untreated cells are symmetrically spread, whereas treated cells show a more rounded morphology and generally appear more asymmetric. Note the membrane extensions, cell polarity, and cell body translocation over the 60 minute period in the EGF-treated cells; current movement is indicated by arrows. Bar, 100 μm .

from each condition were superimposed to a common starting point. These 'wind-rose' plots cover 3 orders of magnitude of Amgel coating concentrations from 0.048 $\mu\text{g}/\text{ml}$ to 4.8 $\mu\text{g}/\text{ml}$. As clearly seen, EGF exposure results in longer cell paths and increased cell dispersion. However, the extent of EGF enhancement varies with substratum density, the maximal response is at an intermediate Amgel concentration, and little EGF induction can be seen at the highest Amgel concentration. In the absence of EGF, Amgel concentration also exerts an effect on cell motility, although the effect is much less dramatic.

EGF increases speed while decreasing directional persistence

To define the parameters of individual cell movement in response to EGF, we calculated speed and persistence times for individual cells. In this fashion, we can derive population parameters from the behavior of multiple individual cells. Basal cell speed was essentially constant across Amgel concentrations

covering two orders of magnitude (Fig. 5A). However, the basal speed is significantly greater than zero, with cells moving over 25 $\mu\text{m}/\text{hour}$. EGF significantly increased cell speed greater than 3-fold, but only at intermediate concentrations of substratum. EGF failed to augment the basal cell motility at the highest concentrations of Amgel. Our data also suggest EGF does not augment basal cell motility at the lowest Amgel concentrations. Examining the population distribution of individual cell speeds demonstrates a single heterogeneous population in both the presence and absence of EGF (Fig. 5B and C). EGF increased both the mean and variance of the population at each 0.048 $\mu\text{g}/\text{ml}$ and 0.48 $\mu\text{g}/\text{ml}$ Amgel concentration.

The effect of substratum concentration on basal persistence was surprising (Fig. 6A). Persistence was at least mildly influenced by Amgel concentration, with an intermediate substratum concentration (0.48 $\mu\text{g}/\text{ml}$) yielding a greater persistence time in the absence of EGF. EGF decreased persistence time in the inverse biphasic manner depending on

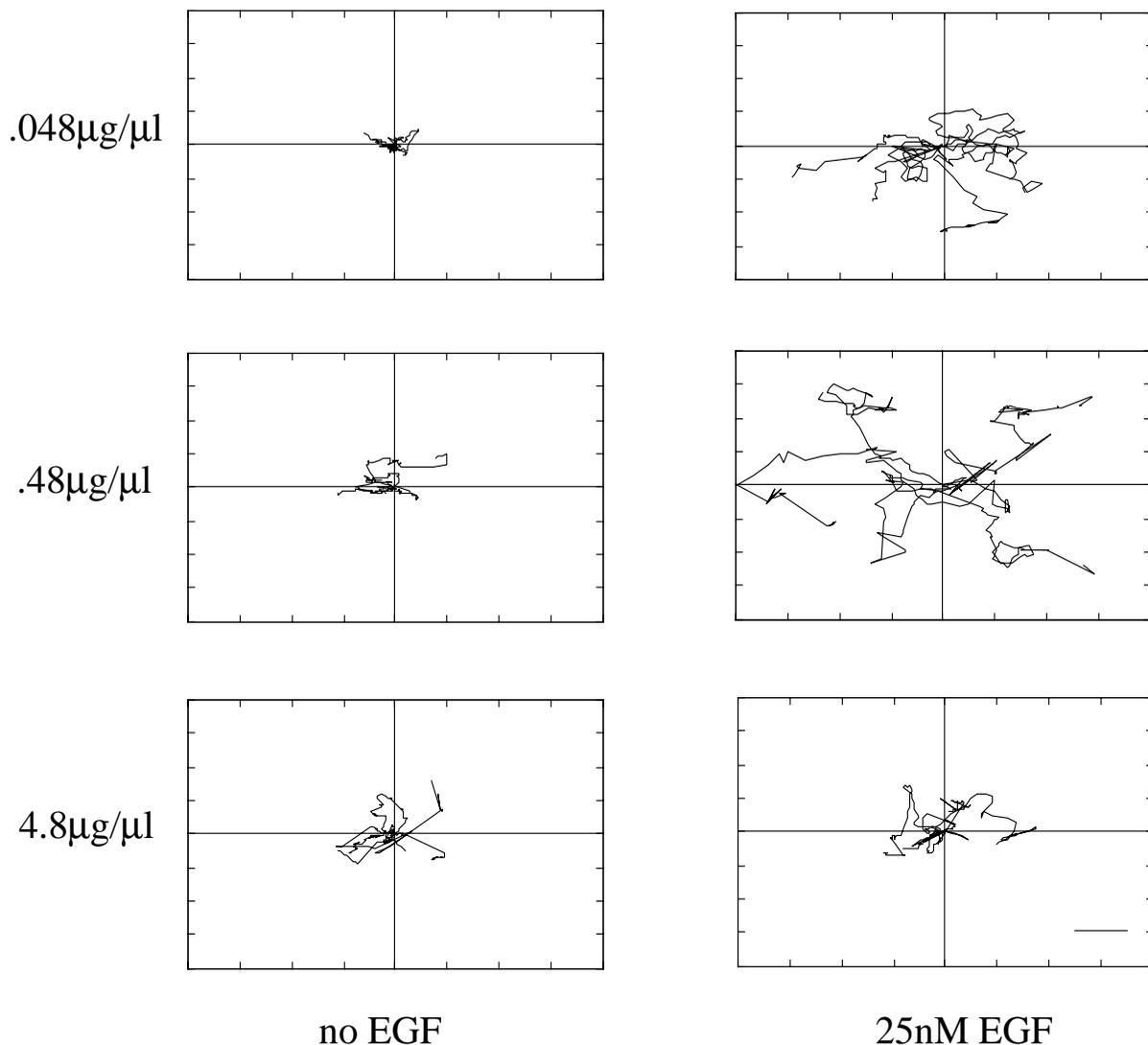


Fig. 4. WT EGFR-expressing NR6 cell migratory behavior is affected by both matrix and EGF. Cell tracks were determined by time-lapse videomicroscopy. Each plot presents representative cell tracks for five cells, with the origins of migration superimposed at 0,0 for clarity of presentation. The cells all express the WT EGFR. Tracks were acquired after more than 8 hours EGF incubation. Distance between hatch marks on both axes is 50 μm .

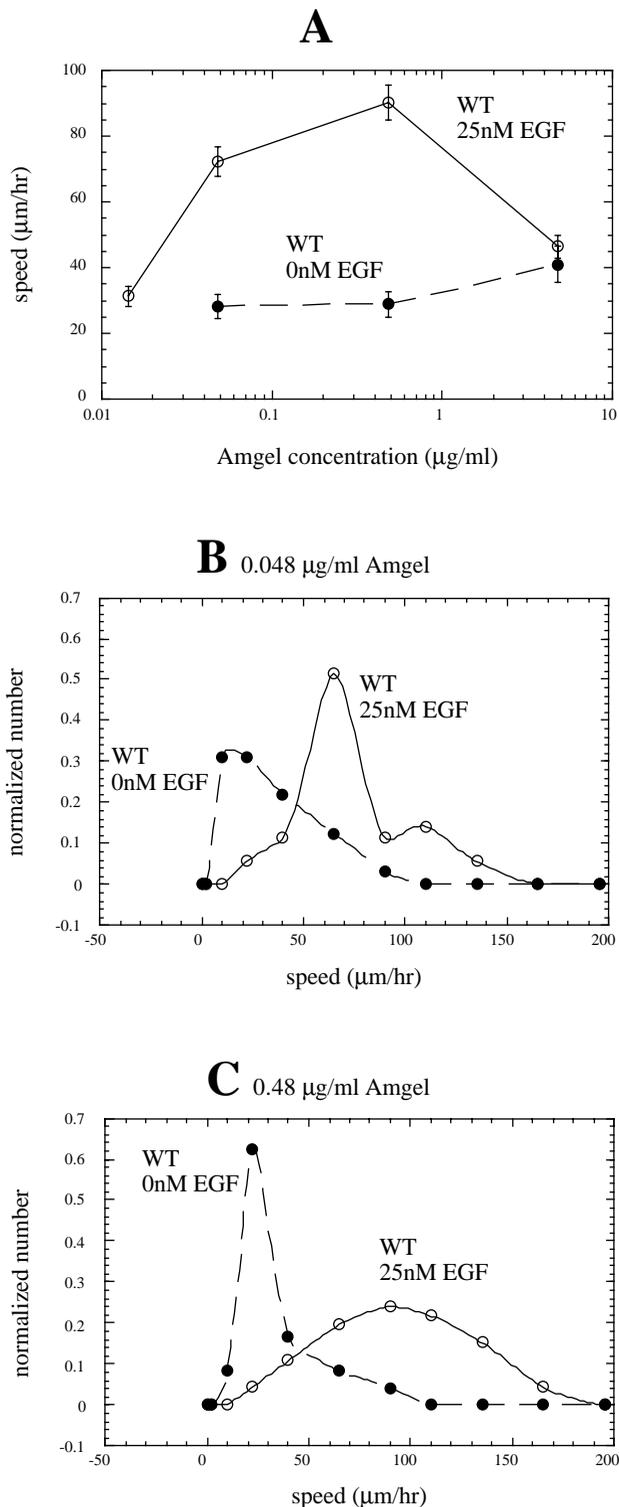


Fig. 5. EGF increases the speed of WT EGFR-expressing cells. Effect of EGF on cell speed was determined for overall population of cells (A) and individual cells (B,C). (A) Mean speeds are reported for a variety of substratum concentrations. Error bars represent the 95% confidence interval based on the standard error of the mean. Cell speed histograms at (B) 0.048 μg/ml ($n=35$) and (C) 0.48 μg/ml ($n=46$) Amgel. The number of cells with a given speed is divided by the total number of cells in the population to give the fractional number of cells at a given speed.

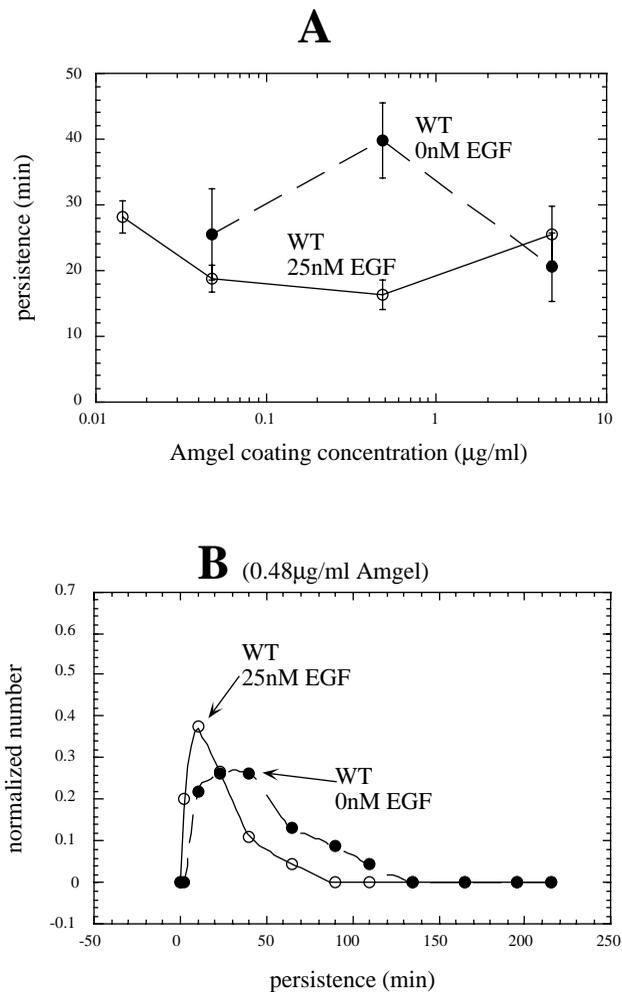


Fig. 6. EGF alters persistence time at an intermediary level of substratum for WT EGFR-expressing cells. Effect of EGF on persistence time was determined for overall population of cells (A) and individual cells (B). (A) Mean persistence times are reported for a variety of substratum concentrations. Error bars represent the 95% confidence interval based on the standard error of the mean. (B) Histogram of cell persistence at 0.48 μg/ml Amgel. The number of cells with a given persistence is divided by the total number of cells ($n=46$) in the population to give the normalized number.

Amgel concentration, accentuating the differences at the intermediary concentrations of Amgel. Again, the cells behaved as a single population, with EGF shifting the the persistence time histogram to the left and decreasing the variability of the response at the concentration of Amgel (0.48 μg/ml) at which a significant difference in persistence is noted (Fig. 6B).

The combination of the motility parameters of speed and persistence determine how far and where the cells migrate (Lauffenburger and Linderman, 1993). The path length ($S \times P$), or distance a cell travels before changing direction, is rather constant over the entire range of Amgel concentrations both in the presence and absence of EGF (Fig. 7A). EGF enhances path length only slightly despite large increases in cell speed; this is due to EGF's reciprocal concomitant effects of increased speed and decreased persistence. Dispersion ($S^2 \times P$), and thus distribution of a cell population, is affected more by changes

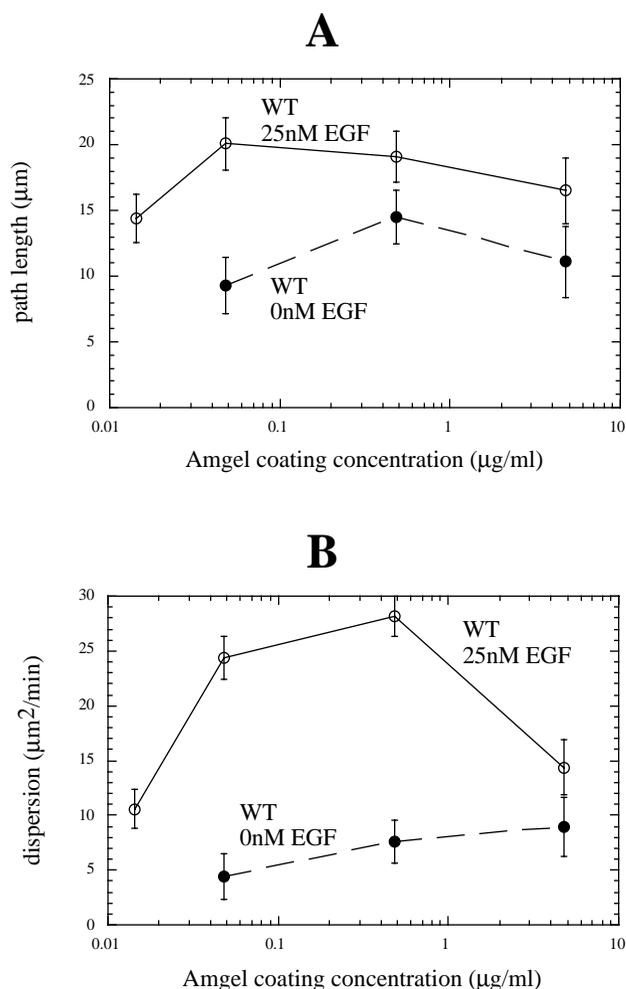


Fig. 7. EGF increases cell dispersion. The effect of EGF on cell path length, SP (A), and cell dispersion, S^2P (B) was determined over different substratum concentrations. Individual cell path lengths and dispersions are calculated and the mean for each condition is reported. Error bars represent the 95% confidence interval based on the standard error of the mean ($n=23-56$).

in speed than in persistence (Fig. 7B). Thus, EGFR activation results in an impressive scattering of cells, as can be appreciated in Fig. 4. Even though EGF causes the cells to turn more frequently through a decreased persistence time, their final distribution away from their starting point is much greater.

EGF decreases cell spread area but increases specific membrane extension rate

We determined growth factor-induced membrane extension in an initial attempt to correlate a specific biophysical process with the noted enhanced cell speed. Membrane extension was investigated as acute exposure to EGF has been shown to induce membrane ruffling (Diakonova et al., 1995; Ridley et al., 1992) and lamellipodial extension (Segall et al., 1996). However, the long-term effect of EGF on membrane activity during sustained cell migration has not been fully elucidated. We measured membrane extension at a time relevant to our EGF-induced cell migration measurements, >8 hours after addition of EGF. At earlier times, within one hour of exposure

Table 1. EGF effects on cell spread area (A), absolute membrane extension rate (B), and specific membrane extension rate (C)

(A) Cell spread area (μm^2)

Amgel concentration ($\mu\text{g/ml}$)	0 nM EGF	25 nM EGF
0.048	1,040±130	690±40
0.48	1,190±160	700±60
4.8	910±70	870±80

(B) Absolute extension rate ($\mu\text{m}^2/15$ minutes)

Amgel concentration ($\mu\text{g/ml}$)	0 nM EGF	25 nM EGF
0.048	260±20	300±30
0.48	300±50	240±20
4.8	340±30	340±30

(C) Specific extension rate (unitless)

Amgel concentration ($\mu\text{g/ml}$)	0 nM EGF	25 nM EGF
0.048	0.25±0.05	0.42±0.08
0.48	0.25±0.07	0.34±0.07
4.8	0.37±0.08	0.39±0.08

The values in C are calculated by dividing those in B by those in A. The data represent between 13 and 20 cells observed over 2 or 3 experiments. Each cell was observed for 4 to 8 15 minute time intervals.

to EGF, cells rounded up and showed a great deal of membrane activity; after this initial burst of activity, the membrane activity quieted and the cells re-spread and began migrating. The migrating cells exposed to EGF exhibited a smaller spread area than control cells (Table 1A), possibly representing an increased ratio of intracellular contractile force to cell/substratum adhesive strength (Welsh et al., 1991). At the highest Amgel concentration, EGF had no effect on cell spread area as noted earlier for cell speed. These data suggest a link between changes in cellular morphology and motility which is supported by the negative correlation between cell speed and spread area (Fig. 8A), and a positive correlation between persistence and cell spread area (Fig. 8B). EGF did not alter the absolute rate of membrane extension (Table 1B). Absolute membrane extension was greatest for cells plated on the highest density of Amgel, suggesting that signals from the matrix contribute to this biophysical phenomenon. Coupled with a decreased cell area, EGF exposure led to an increase in specific membrane extension (membrane extension normalized to cell spread area) at Amgel concentrations that support EGFR-mediated enhanced cell motility (Table 1C). Thus, specific membrane extension is promoted by both growth factor and substratum signals.

In order to determine if membrane extension is a physical mechanism governing EGF-induced cell motility, speed and persistence were each plotted versus specific membrane extension. Specific extension rates appear to exist in distinct groups, one of low extension rates (0.250 ± 0.001) and the other at high rates (0.381 ± 0.028) (Fig. 9A,B). Low extension rates correspond to low speeds and high persistence times and vice versa. There is a positive correlation between speed and specific membrane extension and a negative correlation between persistence time and specific membrane extension.

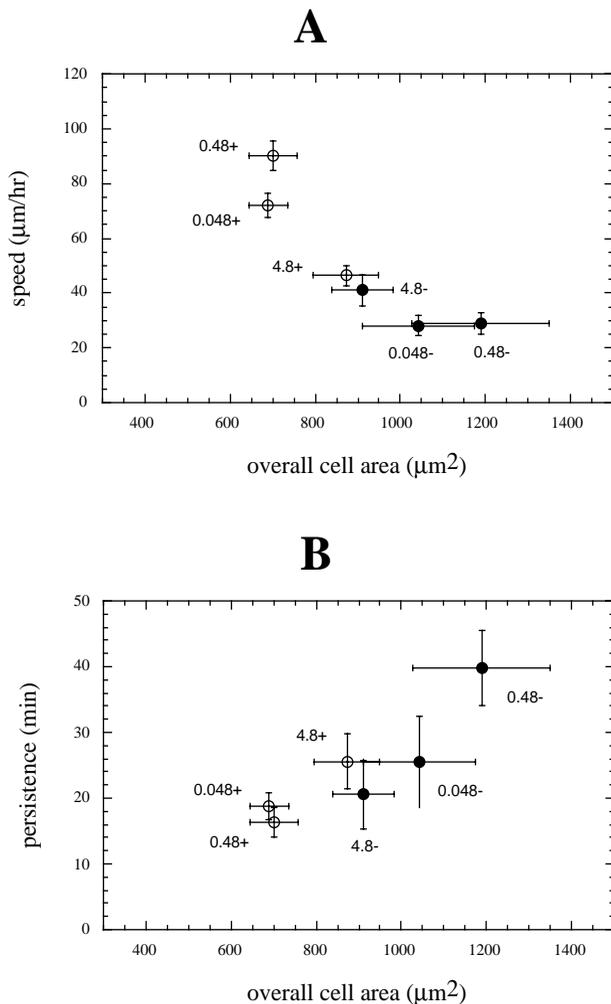


Fig. 8. Dependence of cell speed (A) and persistence (B) on overall cell area. Open circles represent cells treated with 25 nM EGF and closed circles represent cells without EGF, each point represents a different substratum concentration. Numbers denote the Amgel concentration for each data point, along with – or + EGF. Error bars represent the 95% confidence interval based on the standard error of the mean.

DISCUSSION

Activation of receptors with intrinsic tyrosine kinase activity, such as those for EGF, PDGF, and IGF-1, results in enhanced cell motility (Bornfeldt et al., 1994; Chen et al., 1994a; Kundra et al., 1994). While a number of specific biochemical pathways have been shown to be required for this motility response (Chen et al., 1994b, 1996; Ridley et al., 1995), the biophysical characteristics of growth factor-induced sustained cell migration have yet to be well defined. We have described the effect of EGF receptor activation on the foundational motility parameters of intrinsic cell speed and persistence of directional motility.

Two emerging concepts in the field of cell motility are the adhesion dependence of motility and crosstalk between adhesion and growth factor receptors. It is now emerging that haptokinetic movement signaled via integrins follows a biphasic response based upon the adhesiveness to the

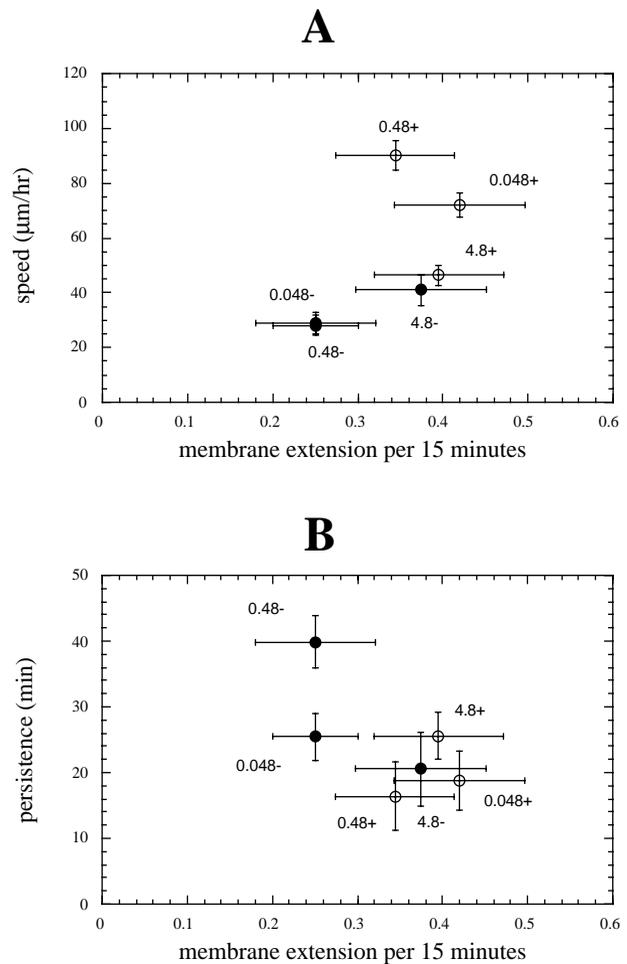


Fig. 9. Dependence of cell speed (A) and persistence (B) on membrane extension rate. Open circles represent cells treated with 25 nM EGF and closed circles represent cells without EGF, each point represents a different substratum concentration. Numbers denote the Amgel concentration for each data point, along with – or + EGF. Error bars represent the 95% confidence interval based on the standard error of the mean.

substratum (DiMilla et al., 1993; Palecek et al., 1997). Previously, EGFR-mediated motility has been examined without considering the density of underlying substrata (Blay and Brown, 1985; Chen et al., 1994a,b; Matthey et al., 1993; Wilson et al., 1994). We determined that the EGF response is very dependent upon the coating substratum concentration. The EGF response is not simply additional to basal motility at each Amgel concentration. Rather, there is a measured response, dependent upon the initial concentration of the underlying substratum (Fig. 5). The fact that EGF-induced motility is noted over only a certain range of Amgel concentrations likely implies that the matrix presents signals both permissive and inhibitory to the EGF motility response. One plausible model requires a certain level of a permissive signal, reached at 0.048 $\mu\text{g/ml}$ Amgel that is effectively negated when a threshold level of an inhibitory signal is reached at 4.8 $\mu\text{g/ml}$ Amgel. Interestingly, we did not note a similar biphasic response for the basal motility. As we tested our cells on Amgel, a complex matrix which contains both

adhesive and antiadhesive signals (Siegel et al., 1993), the lack of a biphasic basal motility may represent an inherent balancing of adhesion and force generation in complex matrices. While it is possible that the surface the cells migrated on was altered from the coating concentration due to nonspecific adsorption of proteins, the reproducibility of the noted substratum dependency strongly suggests that the concepts are valid though the actual substratum density may be somewhat different. Rigorous quantitative analyses of the role of substratum density requires defined linkage to non-adsorptive surfaces; such technologically challenging experiments are planned for future investigations. Further experimentations on singular defined substrata are required to determine whether growth factor-induced motility is governed by specific adhesion-related signals other than adhesiveness in a manner unique to growth factor-induced motility and not integrin-signaled locomotion.

We determined two foundational parameters of motility, speed and persistence time, to gain mechanistic insight into EGFR-mediated cell migration. Other commonly employed techniques for examining cell movement, including Boyden chamber, in vitro 'wound healing' and cell scattering assays, give a general idea of migratory potential but fail to fully describe key parameters and activities. While during short periods (<30 minutes, Fig. 6), adherent cells appear to move in a straight line, over time they exhibit motion similar to Brownian motion of inert particles (Lauffenburger and Linderman, 1993). Defining individual parameters enables us to determine the operational aspects of cell migration, path length ($S \times P$) and dispersion ($S^2 \times P$). By increasing cell speed concomitant with decreasing persistence time, EGF greatly enhances cell dispersion while affecting path length more mildly (Figs 4 and 7). This situation carries possible implications for the biologic control of cell migration in a multicellular organism. A reasonable interpretation is that the rate of cell entry is intrinsic but the locational distribution is dictated by extrinsic signals; if path length were similarly increased by an initial motility signal cell localization would be more difficult to control. An analogous situation is that of axon growth and guidance, in which elongating axons require external cues for directional extension but are capable of random growth in the absence of such guidance cues (Jay, 1996; Komuro and Rakic, 1995; Tessier-Lavigne and Goodman, 1996). In wound healing, for instance, fibroblasts must migrate in to repopulate the nascent wound; the advantage of high speed but low intrinsic directionality of such migration would allow the fibroblast to efficiently distribute themselves based on cell-cell repulsion and matrix encoded signals. During tumor invasion such a random migration would promote tumor spread by increasing cell distribution to find and transverse a plane of spreading or a barrier membrane defect. Our future investigations into EGF-induced cell speed will focus on signaling pathways downstream of EGFR activation, but studies of cell directionality may include alternative receptors, such as adhesion receptors.

We examined the biophysical ramifications of the EGF-effect as a first step in determining the controlling intracellular signaling mechanisms. Differences between basal and EGF-induced cell movement were mirrored in normalized membrane protrusion rates (Table 1). General trends are apparent: a positive correlation of speed and a negative

correlation of persistence with membrane extension. Both results are consistent with physical intuition. As membrane extension increases, overall cell speed also increases as the membrane extension itself represents cell displacement. Concurrently, with more active and rapid membrane extension are increased chances for the cell to change direction and, thus, its persistence time should decrease. Interestingly, while absolute membrane extension was relatively unchanged by EGF exposure, cell spread area was reduced (Table 1). These results are also consistent with physical intuition, with spread area related to the ratio of cell/substratum adhesive strength to intracellular contractile force. EGF thus could decrease this ratio by reducing adhesiveness and/or enhancing force generation (Welsh et al., 1991).

These findings suggest that EGFR-mediated stimulation of sustained cell migration involves regulation of key underlying physical processes, an implication mirrored by biochemical studies which have indicated multiple signaling pathways in growth factor receptor-mediated motility (Bornfeldt et al., 1995; Chen et al., 1994b; Kundra et al., 1994; Ridley and Hall, 1992; Ridley et al., 1992; Wennstrom et al., 1994; Xie et al., 1998). EGFR-mediated cell motility affects both speed and persistence independently, and impinges on cell spread area while not altering absolute membrane extension area. Furthermore, there is a significant effect of substratum density on the EGF responsiveness of cells that is not reflected in the basal, likely adhesion receptor-mediated, cell motility. The value of these biophysical findings is that they construct a rich model of cell migration control, a model which suggests numerous testable hypotheses for future work.

This work was supported by NIH Grant CA69213 to D.A.L. and an NSF Biotechnology Program grant to A.W. Excellent technical assistance was provided by Douglas Osborne.

REFERENCES

- Aaronson, S. A. (1991). Growth factors and cancer. *Science* **254**, 1146-1153.
- Ando, Y. and Jensen, P. J. (1993). Epidermal growth factor and insulin-like growth factor I enhance keratinocyte migration. *J. Invest. Dermatol.* **100**, 633-639.
- Blay, J. and Brown, K. D. (1985). Epidermal growth factor promotes the chemotactic migration of cultured rat intestinal epithelial cells. *J. Cell. Physiol.* **124**, 107-112.
- Bornfeldt, K. E., Raines, E. W., Nakano, T., Graves, L. M., Krebs, E. G. and Ross, R. (1994). Insulin-like growth factor-1 and platelet-derived growth factor-BB induce directed migration of human arterial smooth muscle cells via signalling pathways that are distinct from those of proliferation. *J. Clin. Invest.* **93**, 1266-1274.
- Bornfeldt, K. E., Graves, L. M., Raines, E. W., Igarashi, Y., Wayman, G., Yamamura, S., Yatomi, Y., Sidhu, J. S., Krebs, E. G., Hakomori, S.-I., et al. (1995). Sphingosine-1-phosphate inhibits PDGF-induced chemotaxis of human arterial smooth muscle cells: spatial and temporal modulation of PDGF chemotactic signal transduction. *J. Cell Biol.* **130**, 193-206.
- Cha, D., O'Brien, P., O'Toole, E. A., Woodley, D. T. and Hudson, L. G. (1996). Enhanced modulation of keratinocyte motility by transforming growth factor- α (TGF- α) relative to epidermal growth factor (EGF). *J. Invest. Dermatol.* **106**, 590-597.
- Chakrabarty, S., Rajagopal, S. and Huang, S. (1995). Expression of antisense epidermal growth factor receptor RNA downmodulates the malignant behavior of human colon cancer cells. *Clin. Exp. Metast.* **13**, 191-195.
- Chen, P., Gupta, K. and Wells, A. (1994a). Cell movement elicited by epidermal growth factor receptor requires kinase and autophosphorylation but is separable from mitogenesis. *J. Cell Biol.* **124**, 547-555.
- Chen, P., Xie, H., Sekar, M. C., Gupta, K. B. and Wells, A. (1994b).

- Epidermal growth factor receptor-mediated cell motility: phospholipase C activity is required, but MAP kinase activity is not sufficient for induced cell movement. *J. Cell Biol.* **127**, 847-857.
- Chen, P., Murphy-Ullrich, J. and Wells, A.** (1996). A role for gelsolin in actuating EGF receptor-mediated cell motility. *J. Cell Biol.* **134**, 689-698.
- Chen, Z., Fisher, R. J., Riggs, C. W., Rhim, J. S. and Lautenberger, J. A.** (1997). Inhibition of vascular endothelial growth factor-induced endothelial cell migration by ETS1 antisense oligonucleotides. *Cancer Res.* **57**, 2013-2019.
- Chicoine, M. R. and Silbergeld, D. L.** (1995). The in vitro motility of human gliomas increases with increasing grade of malignancy. *Cancer* **75**, 290-299.
- Diakonova, M., Payrastra, B., van Velzen, A. G., Hage, W. J., van Bergen en Henegouwen, P. M. P., Boonstra, J., Cremers, F. F. M. and Humbel, B. M.** (1995). Epidermal growth factor induces rapid and transient association of phospholipase C-g1 with EGF-receptor and filamentous actin at membrane ruffles of A431 cells. *J. Cell Sci.* **108**, 2499-2509.
- Dickinson, R. and Tranquillo, R. T.** (1993). Optimal estimation of cell movement indices from the statistical analysis of cell tracking data. *AICHe J.* **39**, 1995-2010.
- DiMilla, P. A., Stone, J. A., Quinn, J. A., Albelda, S. M. and Lauffenburger, D. A.** (1993). Maximal migration of human smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. *J. Cell Biol.* **122**, 729-737.
- Duband, J.-L., Dufour, S., Yamada, S., Yamada, K. and Thiery, J.-P.** (1991). Neural crest cell locomotion is induced by antibodies to $\beta 1$ integrins: A tool for studying the roles of substratum molecular avidity and density in migration. *J. Cell Sci.* **98**, 517-532.
- Dunn, G. A.** (1983). Characterising a kinesis response: Time averaged measures of cell speed and directional persistence. *Agents and Actions [Suppl.]* **12**, 14-33.
- Engbraaten, O., Bjerkvig, R., Pedersen, P. H. and Laerum, O. D.** (1993). Effects of EGF, bFGF, NGF and PDGF(bb) on cell proliferative, migratory and invasive capacities of human brain-tumour biopsies *in vitro*. *Int. J. Cancer* **53**, 209-214.
- Gail, M. H. and Boone, C. W.** (1970). The locomotion of mouse fibroblasts in tissue culture. *Biophys. J.* **10**, 980-993.
- Goodman, S., Risse, G. and von der Mark, K.** (1989). The E8 subfragment of laminin promotes locomotion of myoblasts over extracellular matrix. *J. Cell Biol.* **109**, 799-809.
- Jay, D. G.** (1996). Molecular mechanisms of directed growth cone motility. *Persp. Dev. Neurobiol.* **4**, 137-145.
- Klemke, R. L., Cai, S., Giannini, A. L., Gallagher, P. J., deLanerolle, P. and Cheresch, D. A.** (1997). Regulation of cell motility by mitogen-activated protein kinase. *J. Cell Biol.* **137**, 481-492.
- Komuro, H., and Rakic, P.** (1995). Dynamics of granule cell migration: A confocal microscopic study in acute cerebellar slice preparations. *J. Neurosci.* **15**, 1110-1120.
- Kundra, V., Escobedo, J. A., Kazlauskas, A., Kim, H. K., Rhee, S. G., Williams, L. T. and Zetter, B. R.** (1994). Regulation of chemotaxis by the platelet-derived growth factor receptor- β . *Nature* **367**, 474-476.
- Lauffenburger, D. A. and Linderman, J. J.** (1993). *Receptors: Models for Binding, Trafficking, and Signaling*. Oxford University Press, New York.
- Lauffenburger, D. A. and Horwitz, A. F.** (1996). Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369.
- Li, X. B., Nagayasu, H., Hamada, J., Hosokawa, M. and Takeichi, N.** (1993). Enhancement of tumorigenicity and invasion capacity of rat mammary adenocarcinoma cells by epidermal growth factor and transforming growth factor-beta. *Jpn J. Cancer Res.* **84**, 1145-1149.
- Maldonado, B. A. and Furcht, L. T.** (1995). Epidermal growth factor stimulates integrin-mediated cell migration of cultured human corneal epithelial cells on fibronectin and arginine-glycine-aspartic acid peptide. *Invest. Ophthalmol. Visual Sci.* **36**, 2120-2126.
- Matthay, M. A., Thiery, J. P., Lafont, F., Stampfer, F. and Boyer, B.** (1993). Transient effect of epidermal growth factor on the motility of an immortalized mammary epithelial cell line. *J. Cell Sci.* **106**, 869-878.
- Nelson, J., Allen, W. E., Scott, W. N., Bailie, J. R., Walker, B., McFarren, N. V. and Wilson, D. J.** (1995). Murine epidermal growth factor (EGF) fragment (33-42) inhibits both EGF- and laminin-dependent endothelial cell motility and angiogenesis. *Cancer Res.* **55**, 3772-3776.
- Othmer, H. G., Dunbar, S. R. and Alt, W.** (1988). Models of dispersal in biological systems. *J. Math. Biol.* **26**, 263-298.
- Palecek, S. P., Loftus, J. C., Ginsburg, M. H., Lauffenburger, D. A. and Horwitz, A. F.** (1997). Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature* **385**, 537-540.
- Pruss, R. M. and Herschman, H. R.** (1977). Variants of 3T3 cells lacking mitogenic response to epidermal growth factor. *Proc. Nat. Acad. Sci. USA* **74**, 3918-3921.
- Radinsky, R., Risin, S., Fan, D., Dong, Z., Bielenberg, D., Bucana, C. D. and Fidler, I. J.** (1995). Level and function of epidermal growth factor receptor predict the metastatic potential of human colon carcinoma cells. *Clin. Cancer Res.* **1**, 19-31.
- Reddy, C. C., Wells, A. and Lauffenburger, D.** (1994). Proliferative response of fibroblasts expressing internalization-deficient EGF receptors is altered via differential EGF depletion effects. *Biotech. Progr.* **10**, 377-384.
- Ridley, A. J. and Hall, A.** (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389-399.
- Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D. and Hall, A.** (1992). The small GTP binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401-410.
- Ridley, A. J., Comoglio, P. M. and Hall, A.** (1995). Regulation of scatter factor/hepatocyte growth factor responses by ras, rac, and rho in MDCK cells. *Mol. Cell Biol.* **15**, 1110-1122.
- Segall, J. E., Iyerch, S., Boselli, L., Masseling, S., Helft, J., Chan, A., Jones, J. and Condeelis, J.** (1996). EGF stimulates lamellipod extension in metastatic mammary adenocarcinoma cells by an actin-dependent mechanism. *Clin. Exp. Metast.* **14**, 61-72.
- Siegal, G. P., Wang, M.-H., Rinehart, C. A., Kennedy, J. W., Goodly, L. J., Miller, Y., Kaufman, D. G. and Singh, R. K.** (1993). Development of a novel human extracellular matrix for quantitation of the invasiveness of human cells. *Cancer Lett.* **69**, 123-132.
- Tessier-Lavigne, M. and Goodman, C. S.** (1996). The molecular biology of axon guidance. *Science* **274**, 1123-1133.
- Thommen, R., Humar, R., Misevic, G., Pepper, M. S., Hahn, A. W., John, M. and Bategay, E. J.** (1997). PDGF-BB increases endothelial migration on cord movements during angiogenesis *in vitro*. *J. Cell. Biochem.* **64**, 403-413.
- Turner, T., Chen, P., Goodly, L. J. and Wells, A.** (1996). EGF receptor signaling enhances *in vivo* invasiveness of DU-145 human prostate carcinoma cells. *Clin. Exp. Metast.* **14**, 409-418.
- Turner, T., VanEpps-Fung, M., Kassis, J. and Wells, A.** (1997). Molecular inhibition of PLC γ signaling abrogates DU-145 prostate tumor cell invasion. *Clin. Cancer Res.* **3**, 2275-2282.
- Ushiro, S., Ono, M., Izumi, H., Kohno, K., Taniguchi, N., Higashiyama, S. and Kuwano, M.** (1996). Heparin-binding epidermal growth factor-like growth factor: p91 activation induction of plasminogen activator/inhibitor, and tubular morphogenesis in human microvascular endothelial cells. *Jpn J. Cancer Res.* **87**, 68-77.
- Wells, A. and Bishop, J. M.** (1988). Genetic determinants of neoplastic transformation by the retroviral oncogene *v-erbB*. *Proc. Nat. Acad. Sci. USA* **85**, 7597-7601.
- Wells, A., Welsh, J. B., Lazar, C. S., Wiley, H. S., Gill, G. N. and Rosenfeld, M. G.** (1990). Ligand-induced transformation by a non-internalizing EGF receptor. *Science* **247**, 962-964.
- Welsh, J. B., Gill, G. N., Rosenfeld, M. G. and Wells, A.** (1991). A negative feedback loop attenuates EGF-induced morphological changes. *J. Cell Biol.* **114**, 533-543.
- Wennstrom, S., Siegbahn, A., Yokote, K., Arvidsson, A. K., Heldin, C. H., Mori, S. and Claesson-Welsh, L.** (1994). Membrane ruffling and chemotaxis transduced by the PBDFF β -receptor require the binding site for phosphatidylinositol 3' kinase. *Oncogene* **9**, 651-660.
- Wijelath, E. S., Carlsen, B., Cole, T., Chen, J., Kothari, S. and Hammond, W. P.** (1997). Oncostatin M induces basic fibroblast growth factor expression in endothelial cells and promotes endothelial cell proliferation, migration and spindle morphology. *J. Cell Sci.* **110**, 871-879.
- Wilson, S. E., He, Y. G., Weng, J., Zieske, J. D., Jester, J. V. and Schultz, G. S.** (1994). Effect of epidermal growth factor, hepatocyte growth factor, and keratinocyte growth factor, on proliferation, motility, and differentiation of human corneal epithelial cells. *Exp. Eye Res.* **59**, 665-678.
- Xie, H., Turner, T., Wang, M.-H., Singh, R. K., Siegal, G. P. and Wells, A.** (1995). In vitro invasiveness of DU-145 human prostate carcinoma cells is modulated by EGF receptor-mediated signals. *Clin. Exp. Metast.* **13**, 407-419.
- Xie, H., Pallero, M. A., Gupta, D., Chang, P., Ware, M. F., Witke, W., Kwiatkowski, D. J., Lauffenburger, D. A., Murphy-Ullrich, J. E. and Wells, A.** (1998). EGF receptor regulation of cell motility: EGF induces disassembly of focal adhesions independently of the motility-associated PLC γ signaling pathway. *J. Cell Sci.* **111**, 615-624.