Kinematics of Migration of Individual Fibroblasts *in Vitro*: Faster in a Group, but More Target-Oriented Alone

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Abstract

During repair of localized damage in a monolayer of cells**,** migrating NIH3T3 fibroblasts exhibit certain kinematic traits that are influenced directly by factors in their environment such as temperature, pH, and particularly the numbers and distributions of neighboring cells. The present study measures the velocity and direction of individual fibroblasts cultured in a monolayer during migration into a damaged region. Specifically, a parameter termed “percent ideal walk” was defined for evaluating the efficiency of the migrating cells, which were divided into two distinct groups based on migration behavior, namely those migrating alone or with up to four cells and those migrating in a large group (more than six cells). The results show significantly lower velocities for cells that migrated alone or in a group of up to four cells, compared to the velocity of the cells that migrated in a group of more than seven cells ($p < 0.05$). Interestingly, cells that migrated alone or in small groups showed a trend of more efficient directional migration, which was on the edge of statistical significance ($p = 0.056$). This may be attributed to the abundance of mechanical and chemical stimuli that migrating cells generate and sense in a larger group.

Keywords: Fibroblasts, NIH3T3 cells, Wound healing assay, Velocity, Mechanobiology

1. Introduction

Cell migration is a key event in numerous biological processes, such as tumor invasion and metastasis, angiogenesis, immune system response, organogenesis, and wound healing. The latter is the process of repair that follows injury to the skin and other soft tissues, and consists of three main phases [1,2]: 1) the inflammatory phase, which occurs immediately following injury and consists of homeostasis, vasodilatation, and phagocytosis; 2) the proliferative phase, also called the granulation stage, in which fibroblast cells below the dermis begin to increase collagen production and migrate into the damaged area; 3) a remodeling phase, in which the preliminary tissue is remodeled into mature tissue and scar tissue. The process of fibroblast migration, which is a key part of the proliferative phase and is the focus of the present study, is of great interest from the aspect of basic science as well as in the context of developing medications for improving the healing of acute and chronic wounds.

From a bioengineering perspective, the characteristics of cell migration in a monolayer are mostly described by random walk models, some of which model a biased random walk to describe chemotaxis or durotaxis phenomena. The mean cell velocity ranges anywhere between 0-2 μm/min, depending on factors such as the type, stiffness, and surface roughness properties of the substrate, the age (passage number) of the cells, and the biochemistry of the environment, e.g., the pH and presence of epidermal or other growth factors [1-4]. Two factors have been proposed to contribute to the process by which individual cells migrate, called polarization. One involves the generation of a new plasma membrane at the leading edge of the motile cell upon the removal of the plasma membrane at other regions. This permits the extension of the cell surface in a given direction. The other suggested factor is the exertion, by one or more cytoskeletal elements inside the cell, of a force directed at the leading edge [1-4]. This mechanism is assumed to take place as a result of the sensing of mechanical loads acting in the cell’s environment, which can be described, for example, as the strain energy density distribution in the substrate around the migrating cell [5]. Furthermore, it has been suggested that cell-substrate adhesion sites act like local mechanosensors and convert mechanical forces into biochemical signals [6]. In general, the process of epidermal repair is manifested by the progressive extension of a stratified sheet of cells from the peripheral epidermis across the wound [7]. Two phenomena that are instrumental in this extension are the migration and mitosis of epidermal cells. Mitotic activity, however, is normally not observed at or close to the leading margins of the epidermal sheet or in wound assays, but it does exist in cells distal to the area [8].

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The present study investigates the migration of fibroblasts in a monolayer of cells into a site where mechanically-induced damage occurred. The local damage site in the culture is termed here a "wound" for convenience, though the monolayer model system is far from representing the complexity of wounds and wound healing *in vivo*. Model systems involving fibroblasts that migrate in a monolayer were previously used to study the production and secretion of enzymes such as collagenase [9] and growth factors produced by these cells [10]. Some other studies investigated the effects of electrical signals on cell motility in the context of wound healing [11], the effect of the presence of mastocyte cells on fibroblasts migration [12], the location of Golgi apparatus in migrating fibroblasts [13], and the effect of serum factors that promote the migration of fibroblasts [14], to name a few.

Topman *et al*. [8,15] developed a method that is useful for comparative studies of the effects (either environmental or pharmaceutical conditions) on the kinematics of collective cell migration in a monolayer. In this method, migration is measured as "collective cell migration" by tracking changes in the wound area over time. Although extremely useful for measuring the *en mass* kinematics of cultures, the method cannot be used to determine the kinematic behaviors of individual cells. In the present study, cell-specific velocities and efficiencies of migration of fibroblasts cultured in a monolayer during migration from the leading edge ("front line") into the wound are measured and compared.

2. Methods

NIH3T3 cells were grown and cultured on Petri plates (diameter: 35 mm) inside an incubator at 37 ℃ with 5% CO_2. A culture medium containing 10% fetal bovine serum, 90% Dulbecco's Modified Eagle Medium, 0.1% antibiotics cocktail (100 U/ml Penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B), and 2 mM L-Glutamine was used (all the above materials were purchased from Biological Industries, Israel). The cells were cultured until reaching > 85% confluence, which normally occurred 2-3 days after seeding. When approaching confluence, localized damage was inflicted to the monolayer by squashing the cells with a metallic micro-indenter (diameter: 420 μm). This action created an approximately circular wound (Fig. 1). The culture was then rinsed several times using the culture medium.

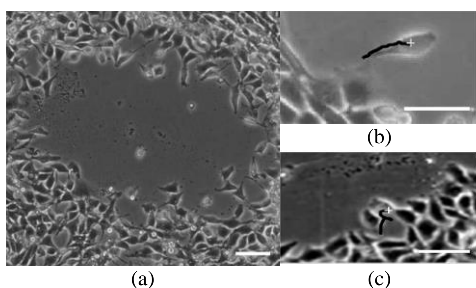

Figure 1. Migration post infliction of the wound. 10X objective; scale bar = 100 μm. (a) Entire wound area. (b) A pioneer cell leaving the front line and migrating alone; the path taken by this cell from the front line is marked. (c) Two cells leaving the front line and migrating towards the wound area together.

Migration of cells into the wound area was monitored using time-lapse microscopy. The temperature of the medium was maintained at 37 ℃ using a heater mat (model 245-635, RS Components Co., UK) and a thermocouple (621-2164, RS Components Co.) connected to a control unit (PXR4, Fuji, 539-5101 RS Components Co.). The pH was kept nearly constant at a level of 7.6 by adding 55 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffering agent) to the culture medium. The cultures were isolated from the environment using a plastic hood while being monitored under the microscope. Micrographs were acquired automatically at 1-min intervals using custom-made software [8,15] and a digital camera (DS-Fi1, Nikon, Sendai, Japan) connected to a phase-contrast microscope (Eclipse TS100, Nikon), whose objective was set to 10X (numerical aperture: 0.3). The resolution of the captured digital micrographs was 2560 × 1920 (3 pixels per micron). Micrographs were captured until complete coverage of the wound was observed, which took about 24 hours.

In order to analyze the captured videos, Time Lapse Analyzer (TLA) software was used [16]. This software, developed in the Matlab environment (The MathWorks, Inc., Natick, MA, USA), is an image processing program specifically designed for tracking cells in time lapse images. For each series of micrographs ($I = 1,2,..,$ number of micrographs), the resulting tracking data, in the form of the center-of-area position vector (***r***) of an investigated cell at micrograph i, was used by TLA for calculating the following parameters:

(i) The mean velocity of a cell, $V_{average}$:

$$V_{average} = \frac{Freq}{N}\sum_{i=1}^{N} r_{i+1} - r_i \tag{1}$$

where $Freq$ is the frequency used for acquiring the micrographs and N is the total number of micrographs taken.

(ii) The "ideal" velocity of a cell, V_{ideal}, from the start point to the end point of the path taken by the cell (i.e., as if the cell was moving along a straight line):

$$V_{ideal} = \frac{r_{end} - r_{start}}{t_{tot}} \tag{2}$$

where r_{start} and r_{end} are the position vectors of the origin and end points of the path, respectively, and t_{tot} is the total time it took the cell to complete movement along the path.

(iii) The "percent ideal walk" (PIW), which is defined in order to quantify the efficiency of motility during the migration of the cells:

$$PIW \equiv 1 - \frac{\left|V_{average} - V_{ideal}\right|}{\sqrt{V_{average}{}^2 - V_{ideal}{}^2}} \tag{3}$$

When a cell advances in a straight line, it takes the shortest path possible to its destination (PIW = unity). Any deviation from this path requires more energy to be consumed to reach the given destination. PIW is hence a simple and straightforward parameter for quantifying this phenomenon. It gives the difference between speeds that the cell could have

used in theory if it had taken the shortest path (V_{ideal}) and its actual average speed ($V_{average}$). Considering a cell that did not travel in a straight line, the speed difference shows how much the cell deviated from the ideal path. Although other well established approaches have been developed to determine cell-cell signaling in the context of cellular injuries [17] and specifically, directionality and directional persistence [18], the above-described straightforward method, which relies on the PIW parameter, was chosen here to quantify directional persistence, mostly because PIW allows simple analysis of large data volumes of cell migration paths.

74 individual cells from a total of twelve experiments were studied. Each cell was sampled once during the entire experiment. When a cell from the leading edge was chosen and monitored, none of its neighboring cells were considered as candidates for monitoring. The studied cells were divided into two separate groups: cells that migrated jointly with more than six other cells in the vicinity[1] (group A) and "pioneer" cells that separated from the front line and entered the wound alone or with up to four other cells (group B). The calculated $V_{average}$, V_{ideal}, and PIW values were statistically compared between groups A and B (using 2- and 1-tailed t-tests for $V_{average}$ and V_{ideal} values and for PIW values, respectively). Significance was set at the 0.05 level.

3. Results

The front line of cells immediately post infliction of the wound and the individual migrating cells were easily identifiable in the time lapse micrographs (Fig. 1(a)). Example images of migrating cells belonging to group B are shown in Figs. 1(b) and (c) and a path taken by a pioneer cell is marked in Fig. 1(b). All tracked cells exhibited migration velocities within the range of velocities reported in the literature [1]. Importantly, the distributions of V_{ideal} and $V_{average}$ data both showed a clear trend of decrease with decreasing number of cells in the vicinity of the tracked cell (Fig. 2). Cells that belonged to group B, that is, the pioneer cells, exhibited significantly lower velocities than those which belonged to group A (those migrating in larger groups) (Fig. 3). Interestingly and importantly, despite being slower, the pioneer cells of group B demonstrated greater PIW values (mean ± standard error: 0.52 ± 0.05) compared to those of group A (mean ± standard error: 0.42 ± 0.04), which was on the edge of statistical significance ($p = 0.056$).

4. Discussion

Following infliction of the wound, stimulatory signals (several of which have been identified in the literature [5,17]) change upon release of contact inhibition. Such mechanical wounding may also eliminate inhibitory signals for migration that are mediated by cell surface proteins, e.g., contact inhibition [17]. Here, it was found that the immediate surroundings of the migrating cell significantly influence its velocity. Pioneer cells that are the first to leave the front line post infliction of the wound (group B) tend to attain a velocity that is significantly lower than that exhibited by cells that migrate in larger groups (group A) (Fig. 3). However, pioneer cells (group B) appear to be more target-oriented in their travel towards the denuded area, and thus have higher PIW values (which were on the limit of being statistically significantly different compared to those of group A).

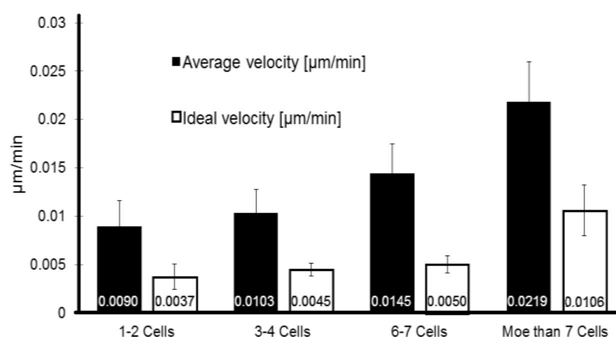

Figure 2. Average and ideal cell velocities as functions of the number of cells in the micro-vicinity of the migrating cell shown to emphasize the trend of increasing velocities with increasing number of surrounding cells.

[1] "Vicinity" is defined here as the radius of the wound in the micrograph

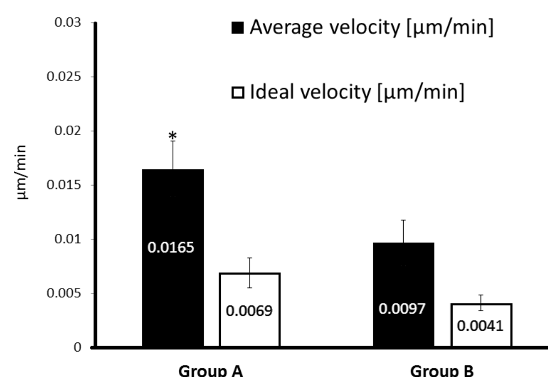

Figure 3. Average and ideal cell velocities of the cell groups. Error bars refer to the standard error around the mean. *$p < 0.05$ and indicates a statistically significant difference between groups A and B.

The results imply that cells which migrate alone do so more efficiently compared to cells that migrate in a large group. Since cells that migrate in larger groups generally take a path that is substantially longer than a straight line for moving from the start to the end point of their trail, the mechanical work that each of these individual cells needs to perform to reach its target is greater than that required by the pioneer cells (work = force times distance; it is assumed that the driving forces are similar). The increased randomness of the migration pattern of the cells belonging to group A is compensated to some extent by them being faster (Fig. 3), and their increased velocity can be explained in terms of the direction and amplitude of the incoming chemical signals from neighboring cells, as previously suggested [6]. Specifically, we refer here to a mechanical field (deformation or strain) previously described in detail [5]. The cells are regularly attached to their substrate. The force they exert on their surroundings is sensed by neighboring cells through the substrate. Considering a group of more than two cells, the force vectors that are received by a certain cell from adjacent cells are summed to produce the total

force vector (i.e., force superposition). Super-positioning the incoming signals for an individual cell from group A, which originate from other cells in its vicinity, results in a high directional variation as well as high signal amplitudes, which promotes faster, but less directionally consistent, movement. In contrast, super-positioning the incoming signals for a cell from group B likely results in a vector that points towards the center of the wound region but at a lower signal amplitude, which induces more directionally consistent, but slower, progress towards the center of the wound.

5. Conclusion

In closure, the findings lead to the conclusion that when cells migrate into the wounded area alone or in very small groups, they are less influenced by mechanical stimuli and chemical stimulatory signals that are transmitted regularly from cells in their immediate surroundings, which allows more directionally efficient migration.

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