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The behavior of chick gastrula mesodermal cells under the unidirectional tractive force parallel to the substrata

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SUMMARY

Advancement of leading lamellae of a migratory cell inevitably causes a strain inside the cell body. We investigated the effect of the tension arisen inside a mesodermal cell on its behavior by pulling the cell body unidirectionally along the substratum.

Chick gastrula mesodermal cells, known as highly migratory, were dissociated into single cells in sodium citrate buffer, conjugated with paramagnetic beads activated by tosyl-residue (4.5 μm in diameter) and seeded onto coverglasses coated with fibronectin. After the cells spread on the substratum and protruded cellular processes in all directions, they were exposed to a non-uniform magnetic field by a magnet. Thus the cells bearing the beads were pulled with a force in the order of 10^{-10} N. The behavior of such cells was recorded with a time-lapse video taperecorder and assessed quantitatively.

Shortly after the magnetic force was applied, the beads stuck to the cells were aligned in tandem along the line of magnetic force at the site for the magnet. Subsequently, they frequently came to extend their leading lamella precisely counter to the traction on the line of the beads. Observation with scanning electron microscope revealed that a large part of the beads attached to the cells were wrapped in the cell membrane. In this condition, the cells were stretched locally between the attachment site of the beads and adhesion plaques beneath the leading edge, which was formed in a direction away from the traction. It was proved statistically that such cells tended to locomote away from the magnet at the 0.1% significance level with Hotelling's T^2-test. In contrast, the mesodermal cells free of the artificial traction in three kinds of control experiments did not show such a preference in the direction of locomotion.

These results proved that migratory cells tended to move in the direction away from the tractive force parallel to the substratum, suggesting that advancement of a leading lamella is accelerated when it is stretched along the direction of projection by a mechanical force of sufficient strength. Implication of this finding to the mechanism of cell locomotion will be discussed.

Key words: traction, locomotion, mesodermal cell, leading lamella, stretch, magnetic bead

INTRODUCTION

In morphogenic movement, most of the embryonic cells are interlinked with one another so tightly that such cells cannot move without giving any mechanical stress to the neighboring cells: when a member of a cell sheet contracts and reduces its surface area, the neighbors are inevitably stretched. It is proposed that traction and resultant directional strain play some regulatory role in the morphogenic movement (Beloussov et al., 1975; Oster et al., 1983). To understand the contribution of the traction to the morphogenesis at the cellular level, we think it necessary to examine the behavior of the embryonic cells in response to the tensile stress in vitro.

Some tissue cells are known to change their shapes and motility under mechanical stress. When vascular endothelial cells or smooth muscle cells surrounding them were cultured on a substratum that was periodically stretched and relaxed, both of them oriented perpendicular to the axis along which the cells were stretched (Buck, 1980, 1983; Dartsch and Hammerle, 1986; Shirinsky et al., 1989). Osteoblasts also aligned perpendicular to the direction of stretching on the elastic substratum (Buckley et al., 1988). Fluid sheer stress, when applied to the endothelial cells, leads the cells to change their shape from polygonal to elliptical, and to be oriented with the flow (Dewey et al., 1981; Franke et al., 1984). When granulocytes were exposed to the mechanical stress by centrifugation, their movement for and against the centrifugal force speeded up (Doroszewski et al., 1986). When stretched along the substratum, corneal epithelial cell sheets started to expand much faster than those without the tension (Takeuchi, 1979).

Concerning the migratory cells in locomotion, they are always exposed to the intrinsic tensile stress which is produced by the advancement of the leading lamellae and succeeding contraction of the elongated elastic cell body (Oster, 1989). However, it is still unknown whether such tensile stress plays a positive role in cell locomotion or not. In this report, we applied artificial extrinsic traction to the migratory cells in vitro in a direction parallel to the substratum, intending to examine the role of the tensile stress. Chick early gastrula (early primitive streak stage) mesodermal cells were chosen as

material in this experiment, because they in situ migrate actively to form the germ layer under the epiblast in the central region of the blastoderm called the area pellucida, which is always stretched to be flat by the contractile area opaca surrounding it (Kucera and Monnet-Tschudi, 1987).

Chick early gastrula mesodermal cells, dissociated into single cells and conjugated with paramagnetic polystyrene beads, were cultured on the substratum. Then a magnet was positioned aside. Thus the cells were pulled by the beads unidirectionally parallel to the substratum. It was proved statistically that the cells tended to move in the direction away from the magnet in this condition. Based on these results, we will discuss the possible mechanism of this novel behavior of the migratory cells and its implication to the mesoderm layer expansion.

MATERIALS AND METHODS

White leghorn chicken eggs were incubated at 37°C for 20 hours until embryos had reached stage 3^+ of Hamburger and Hamilton's table (1951). The whole blastoderm was excised in phosphate buffered saline (PBS) and the inner layer (mainly hypoblast) was removed with a glass needle. The small fragments of mesodermal cells containing 20-200 cells were dissected out from both sides of the primitive streak near Hensen's node with the needle and transferred into a droplet (50 l) of HEPES buffered Ca^{2+}, Mg^{2+}-free Tyrode solution (pH 7.2) containing 20 or 40 mM sodium citrate on a paraffin bed. Then the fragments were pipetted gently every 5 minutes for 15 minutes. In dissociation with enzymatic digestion, the mesodermal cells lost their activity of attaching to the fibronectin substratum. As for our procedure, approximately 90% of the chick early gastrula mesodermal cells (4,660 out of 5,198 cells) were isolated as single cells, and the cells kept their activity to spread on the substratum.

Conjugation of paramagnetic beads with the cells

Paramagnetic beads (4.5 μm in diameter) modified with tosyl residue (Dynabeads M-450 Tosyl-activated; Dynal Co., Oslo, Norway) were suspended in the droplets containing the mesodermal fragments. Then the fragments were pipetted gently as above, for the cells to be dissociated and come into contact with the beads. Probably because of the covalent bonds formed between tosyl residues on the beads and the cell surface, the beads attached firmly to the cell surface (Fig. 1). When seeded onto the substratum, almost all the single mesodermal cells, even bearing the beads, attached to the fibronectin substratum. We tried to use the non-coating 'Dynabeads', but they hardly attached to the cell surface; only a few beads attached to the cells, but for a short period.

Cell culture

Medium 199 supplemented with 20% chicken serum (Cosmo Bio Co., Japan) and buffered with 3 mM HEPES (pH 7.6) was used. In some cases, fetal bovine serum (FBS, Gibco) was used instead of the above serum. The mesodermal cells either carrying the beads or not were seeded onto the surface of the coverglass (24 mm × 24 mm) coated with bovine plasma fibronectin (50 mg/ml; Biomed. Tech. Inc., USA) for 1 hour at 37°C. The number of cells collected from one embryo was suitable for each coverglass, giving a cell density low enough to avoid cell-to-cell contact. In 30 minutes of preincubation at 37°C in 5% CO_2-95% air, the cells attached to and spread flat on the coverglass. Then the coverglass was turned upside down and settled on an observation chamber made of silicone rubber and a slide glass (5 mm in thickness) filled with fresh medium, so that the cells were submerged in the medium. During this procedure, almost all the beads attached loosely to the surface of the coverglass were removed.

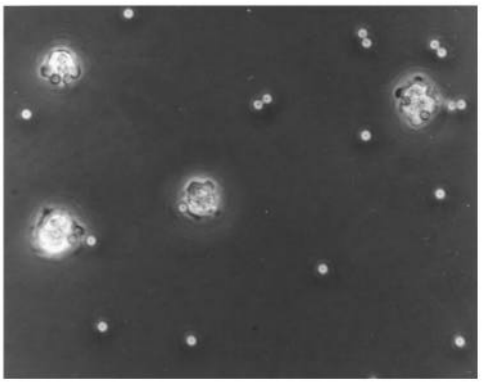

Fig. 1. Phase contrast micrograph of mesodermal cells conjugated with paramagnetic beads just after being seeded onto the fibronectin substratum. Note that the cells were dissociated into single cells carrying several beads in each.

Application of magnetic force

A rectangular neodymium magnet block (Br= 1.18 (T), Hcb= 900 (kA/m); 10 mm (height) × 24 mm (width) × 40 mm (length), magnetized in the direction along which the length was measured (TDK Co., Japan)) was placed so that the magnetic pole face was in touch with one side wall of the observation chamber (5 mm in height). In this condition, every bead positioned on an extrapolated line of the axis of magnetization was pulled by the magnet in the direction correctly parallel to the substratum (Fig. 2a).

At the point 15 mm apart from the magnet pole face on the center magnetic line of force, the intensity of the magnetic force (H) was estimated as:

$Hx=4.72\times10^4$ (A/m), and the gradient, $dHx/dx=5.28\times10^6$ ((A/m^2); x=distance from the magnet). Thus, $Hx\times dHx/dz=0.394$ (T^2/m). According to the information from Dynal Co., Dynabeads M-450 show superparamagnetic behavior, and the magnetic susceptibility was approximately $4\pi \times10^{-2}$ in the condition of $Hx\times dHx/dz=0.335\pm0.005$ (T^2/m). Based on these values, the tractive force worked on a cell was calculated to be approximately 10^{-10} N.

Observation and recording of cell behavior

A phase contrast microscope (Nikon, Japan) equipped with a TV lens (Nikon) and connected with a time-lapse video taperecorder (JVC, Japan) was used. The stage of the microscope was kept at 37°C by blowing warmed air.

The behavior of the cells were observed and recorded for more than 30 minutes under four kinds of the conditions as follows:

Exp. 1: having no beads and not being subjected to the magnetic field;

Exp. 2: having no beads and being subjected to the magnetic field;

Exp. 3: having magnetic beads and not being subjected to the magnetic field;

Exp. 4: having the beads and being subjected to the magnetic field, that is, being pulled by the mechanical force.

In these experiments, fully spread cells positioned 8-16 mm apart from the surface of the magnet and on the axis of magnetization were observed and recorded (Fig. 2a). Recording was started after 30 minutes of incubation in the above four cases, on applying the magnetic field in the case of Exp. 2 and Exp. 4. Then the displacement of the cells for 30-120 minutes was processed for quantitative analysis. Whenever the cells in focus came close to the other cells nearer than 30 μm during recording, such data were discarded in order to eliminate the influences of the density effect on cell motility. Artifacts such as immobile fine clots of serum in a field of view were marked for fixed points. The mesodermal cells were more easily damaged by the illumination of the microscope than embryonic

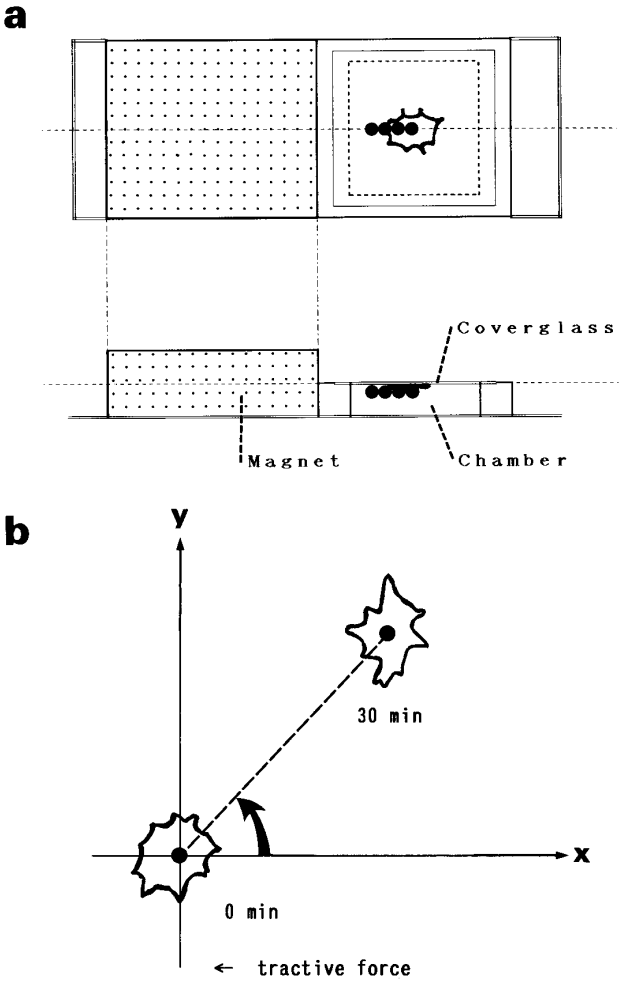

Fig. 2. (a) Illustration showing the setup of the culture chamber equipped with the magnet in order to expose the cells bearing several paramagnetic beads to the magnetic force. For a cell just on the axis of magnetization (dotted line through the center of the magnet), the direction of traction is exactly in parallel with the substratum. (b) Illustration showing the method of quantification of cell locomotion. For each cell, its position at the beginning of recording was standardized to the origin of a scattergram. The *x*-axis was aligned as shown against the direction of the tractive force, and the *y*-axis perpendicular to the *x*-axis. Direction of cell locomotion was measured in degrees anti-clockwise against the line through the axis of magnetization (arrow). We collected data of single cells separated from other cells by more than 30 μm.

fibroblasts in general, so the illuminating power was held down as far as possible. Some photographs were taken with Neopan SS film (ISO=100, Fuji), and the others with a video printer (Mitsubishi, Japan). Video image was improved with the aid of an image processor (Hamamatsu Co., Japan) on print out.

Contact sites of the mesodermal cells to the substratum were observed with an interference reflection microscope (Olympus, Japan), and microphotographed with Tri-X film (ISO 400, Kodak).

Scanning electron microscopy

Immediately after being exposed to the magnetic force in the same condition as Exp. 4 for 60 minutes, the cells were fixed with 2% glutarardehyde in PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$. They were dehydrated, air-dried and coated with gold using an ion coater (JFC-1100E, JEOL). Then the cells were observed with a scanning electron microscope (SEM; T-20, JEOL) and photographed (×3000-×5000).

Quantitative assessment of cell behavior

With the aid of a video image printer (Mitsubishi, Japan), cell figures at the beginning and after 30 minutes were outlined on a sheet of transparent acetate film. The cell center was defined as the gravity of the lattice points dotted inside the outline, which was calculated for each with a digitizer (Graphtec Co., Japan) and a personal computer (NEC, PC-9801RA)(Toyoizumi and Takeuchi, 1992). For each experiment, tips of vectors representing cell locomotion for 30 minutes were plotted as Cartesian coordinates in a scattergram, in which the positions of the cell centers at the beginning of recording were standardized at the origin of the graph. The *x*- and *y*-axes were lined along or perpendicular to the line through the axis of magnetization as shown in Fig. 2b.

For each scattergram, parametric and non-parametric Hotelling's T^2-tests to the second-order samples (see Batschelet, 1978, for details) were applied to judge whether tips of vectors are concentrated to a certain direction or not. The parametric test stands on the assumption that the sample was taken from a bivariate normal distribution. In this method, the null hypothesis is rejected when the confidence ellipse associated with the plots excludes the origin, while the non-parametric alternative can be used even when the above assumption is seriously violated. In addition, a two-tailed *t*-test to two groups based on the null hypothesis assuming that the two groups are taken from the same population was also applied either to the *x*- or *y*-components (components along, and those perpendicular to, the axis of magnetization; see Fig. 2b) of the vectors. Here we used Welch's method. This method is available even when the population variance is unknown and the equivalence of the variances of the two samples is not assured. In this method, test statistic $t(\phi)$ is used, where ϕ is calculated with the equation:

$$\phi = (Sa^2/na + Sb^2/nb)^2/[(Sa^2/na)^2/(na-1) + (Sb^2/nb)^2/(nb-1)]$$

(*na*, *nb*=number of sample; *Sa*, *Sb*=standard deviation of the population '*a*' or '*b*').

RESULTS

Exp. 1: behavior of the cells without the beads, outside the magnetic field

Most of the mesodermal cells without the beads spread uniformly and protrude cellular processes in all directions within 30 minutes of incubation at 37°C (Fig. 3). The spread cells protruded and retracted actively small tongue-like lamellae and many filopodia. Adhesion plaques were formed along the peripheral edges of the cells (Fig. 3). They moved while changing their direction of locomotion incessantly. They sometimes rounded up while forming many blebs, and soon spread again and ceased to bleb, as was observed in mouse gastrula mesodermal cells in vitro (Hashimoto et al., 1987). After a few hours in culture, formation of the cellular processes was confined in a few leading lamellae and the cells began to locomote with a typical crawling movement (Abercrombie, 1980).

A scattergram in Fig. 4a shows the destination of cell locomotion after 30 minutes of recording viewed from the initial position at the beginning of recording. In 52 cells in a total, there was no detectable tendency in the direction of locomotion with parametric or non-parametric Hotelling's T^2-test at the 10% significance level (Fig. 5; Tables 1 and 2). That is, the cells locomoted randomly in all directions, and no preference in the direction of locomotion was detectable.

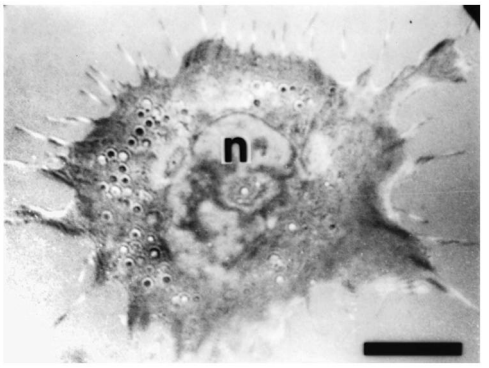

Fig. 3. Interference reflection image to show the distribution of the focal contacts in a mesodermal cell. This cell was seeded onto the substratum coated with fibronectin and incubated for 45 minutes before photographing. Note that the cell spread in a circular form with many fine processes protruding in all directions and that focal contact sites were formed along the most peripheral edges of the cell (dark spots). Artificial interference reflection between the nucleus and the plasma membrane is marked with 'n'. Bar, 10 μm.

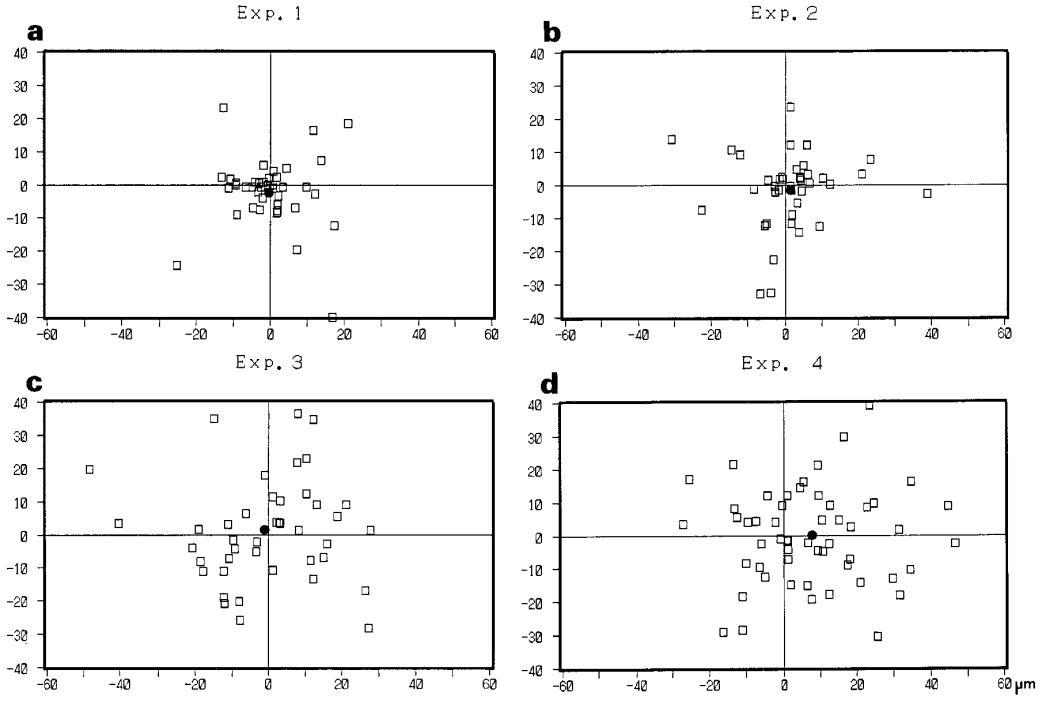

Fig. 4. Scattergrams showing the tips of vectors representing cell locomotion for 30 minutes in Exp. 1 (a), Exp. 2 (b), Exp. 3 (c), and Exp. 4 (d). Closed circles indicate the gravity of the plots. (a) Scattergram of the cell location in Exp. 1 (without the magnetic field and the beads; see Materials and Methods). Number of the plots is 52. (b) Scattergram of the cells in Exp. 2 (having no beads and being subjected to the magnetic field). Number of the plots is 40. (c) Scattergram of the cells in Exp. 3 (having paramagnetic beads and not being subjected to the magnetic field). Number of the plots is 45. (d) Scattergram of the cells under artificial traction parallel to the substratum in Exp. 4 (having paramagnetic beads and being subjected to the magnetic field). Number of the plots is 60. Gravity of the plots indicates that distribution of the plots are concentrated to the plus side of the *x*-axis, a direction counter to the tractive force, which is confirmed statistically with various tests in Fig. 5 and Tables 1, 2, 4 and 5.

Table 1. Hotelling's one-sample T^2-test for the displacement of cell centers (parametric method)

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
n	52	40	45	60
$F_{2, n-2}$	1.20	0.70	0.29	6.86
F_0 (0.01)	5.06	5.21	5.14	4.97
Probability	>0.1	>0.1	>0.1	<0.001
Direction of the center of the confidence ellipse*	−90°	−53°	129°	+0.1°
Position of the center of the confidence ellipse	(0.00, −2.04)	(1.24, −1.63)	(−1.29, 1.63)	(8.01, 0.02)
Distance between the origin and the center of the ellipse	2.0	2.1	2.1	8.0
Velocity of locomotion (μm/hour)	18.9±17.9	25.0±19.9	44.5±30.2	41.2±24.5

Test values of Hotelling's T^2-test and parameters of the confidence ellipses in Fig. 5. Note that only the distribution of the plots in Exp. 4 (Fig. 4d) deviates significantly to a direction (P<0.1%). Position of the center of the confidence ellipse suggests that the direction of this deviation is on the opposite site of the magnet. Note also that when we compare the positions of the center of the ellipses, we find the cell population in Exp. 4 migrated furthest to a direction.

*Angle held between the plus-end of the *x* axis and the line connected between the origin of the graph and center of the confidence ellipse.

Exp. 2: behavior of the cells without the beads, in the magnetic field

Fig. 4b shows the directionality of locomotion of the mesodermal cells with no beads in the magnetic field. Morphology and the behavior of the cells appeared to be the same as for those in Exp. 1. In 40 cells in a total, there was no detectable tendency in the direction of locomotion either with the parametric two-dimensional Hotelling's test or with the non-parametric one at 10% significance level also in this case (Fig. 5; Tables 1 and 2). Speed of locomotion of the cells (25.0±19.9 (μm/hour)) did not differ from that in Exp. 1 (18.9±17.9 (μm/hour)) (Table 3).

Exp. 3: behavior of the cells withthe beads, outside the magnetic field

The beads attached to the cell surface were observed to form a loose cluster at the central part while changing their position incessantly, probably due to the cortical flow of cell membrane at the dorsal surface of the leading lamella (Abercrombie et al., 1970; Dembo and Harris, 1981) (Fig. 6). The cells loaded with more than 10 beads also spread at first, but soon lost their attachment to the substratum and took a spherical form. The cells bearing several beads, however, took a spread form and behaved like the cells in Exp. 1, except in the speed of locomotion.

The speed of locomotion in Exp. 3 was 44.5±30.2 (μm/hour) (mean ± s.d. in 45 cases), which is significantly different from the speed in Exp. 1 or in Exp. 2 (Tables 1 and 3).

As for the directionality, forty-five cells bearing 2-8 beads were analyzed and no preference in the direction of locomotion was detectable with Hotelling's T^2-test or Student's *t*-test even at the 10% significance level (Fig. 5; Tables 1, 2 and 4).

Table 2. Hotelling's one-sample T^2-test for the displacement of cell centers (non-parametric method)

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
n	52	45	40	60
Test value	0.37	1.33	0.34	13.7
Critical value (U_0 (0.1))	4.61	4.60	4.60	4.61
Critical value (U_0 (0.01))	8.9	8.8	8.8	8.9
Probability	>0.1	>0.1	>0.1	<0.01

Non-parametric Hotelling's T^2-test to the scattergrams in Fig. 4. As is the same with Table 1, only the distribution of the plots in Exp. 4 deviates significantly from the origin to a certain direction (P<1%).

Table 3. Welch's *t*-test for the velocity of locomotion among the samples

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Experiment 1		1.50 (ϕ=79) P>0.1	4.93 (ϕ=69) P<0.01	5.50 (ϕ=107) P<0.01
Experiment 2			3.51 (ϕ=77) P<0.01	3.59 (ϕ=94) P<0.01
Experiment 3				0.59 (ϕ=83) P>0.5

In Welch's method, test statistics $t(\phi)$ is used instead of $t(na+nb-1)$ (see Materials and Methods).

Welch's t-test to investigate the significant differences in the velocity of cell locomotion among the four experiments. It is recognized that velocities in Exp. 1 and Exp. 2 form one group and those in Exp. 3 and Exp. 4 form another, and that the latter group is significantly more locomotive than the former.

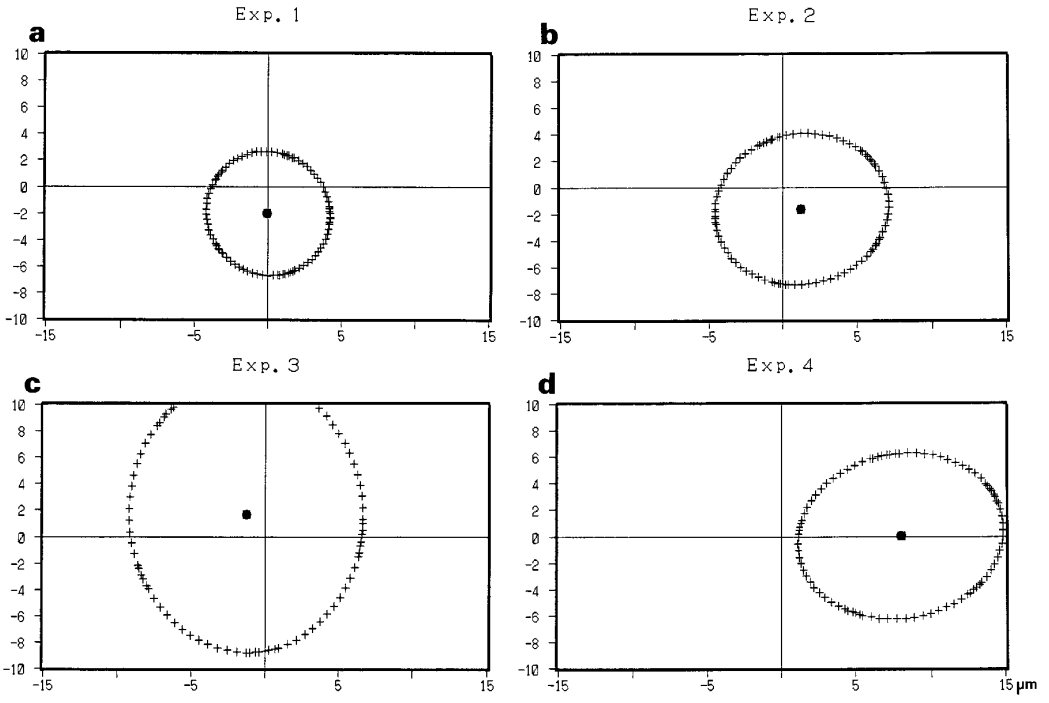

Fig. 5. Hotelling's confidence ellipses showing 99% confidence area corresponding to the scattergrams in Fig. 4. If an ellipse for a given significance level covers the origin of the graph, a concentration of vectors (corresponding to the center of the ellipse; closed circle) around a certain direction is rejected statistically. On the other hand, if the ellipse does not cover the origin, it is concluded that the vectors are somewhat concentrated around a mean direction (Batschelet, 1978). Note that only the ellipse of Exp. 4 (Fig. 4d) excludes the origin. Thus, cell locomotion in Exp. 4 is confirmed to be biased in the direction counter to the tractive force at the 1% significance level. There is no significant preference in the direction of cell locomotion in Expts 1, 2 and 3. Note also that the center of the ellipse of Exp. 4 is most distant from the origin. Details of the statistical values are shown in Table 1.

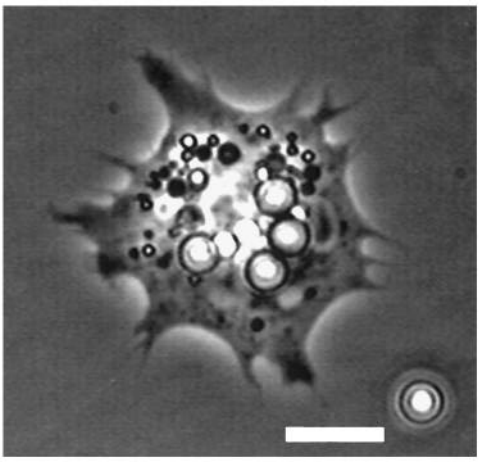

Fig. 6. Phase contrast micrograph of a mesodermal cell bearing paramagnetic beads incubated for 30 minutes without applying the magnetic field in Exp. 3. Four beads are gathered at the center of the cell body probably due to the cortical flow of the plasma membrane. Note that this cell spreads in a circular form with many processes protruding in all directions. Bar, 10 μm.

Exp. 4: behavior of the cells having the beads, in the magnetic field

Sixty cells in total bearing 2-8 beads (2.7 on average) were exposed to the magnetic force. Most of the beads, 169 beads out of 239 (71%) in 47 cells incubated for 60 minutes with exposure to the magnetic force, were phagocytosed when the cells were observed with SEM (Fig. 7). Here we judged a bead to be phagocytosed when more than half of the surface of the bead was covered with cell membrane.

Within the first few minutes of exposure to the magnetic field, paramagnetic beads attached to the cells began to align in tandem along the line of magnetic force. As shown in Fig. 8, the beads attached to the cells usually shifted their position to the magnet, lining up gradually along the magnetic field. Some of the beads, perhaps those weakly attached to the cell surface or to the substratum, were detached from their original position as soon as the magnetic force was applied. Then, such beads flew away to the magnet or came to join a line of the beads near by (see the left cell in Fig. 9). When a cell was loaded with many beads in the magnetic field, the beads were lined up across the cell body as shown in Fig. 9b.

A large part of the cells started to locomote in a direction away from the magnet, that is, against the tractive force (Fig. 9). The

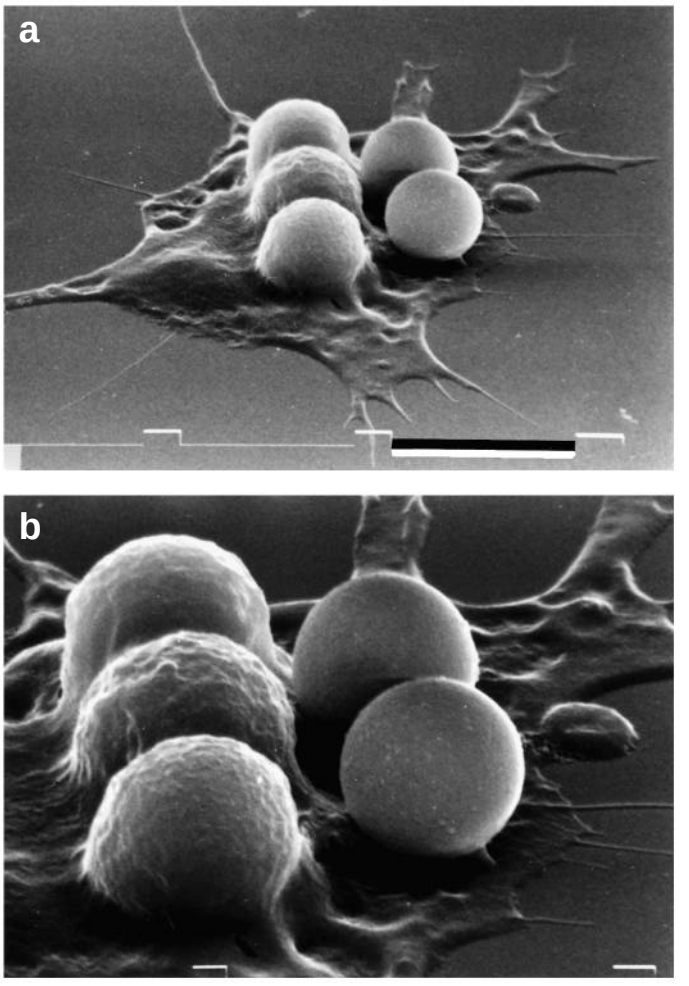

Fig. 7. Scanning electron micrograph of a mesodermal cell having 5 beads that was fixed immediately after having been subjected to the magnetic force for 1 hour. Note that 3 beads out of 5 are recognized to be wrapped in cell membrane and the other two stuck to the cell surface. (a) Low power view. (b) High power view. Bars, 10 μm.

cells showing the escape behavior often extended their leading lamella precisely counter to the force, and took on elongated forms in a direction perpendicular to the traction, transiently but many times, which was never observed in Exp. 3. The cells bearing several beads and showing 'escape behavior from traction' often continued to locomote in a direction away from the magnetic force and drew straight trajectories for more than

Table 4. Two-tailed one-sample Student's *t*-test for the averages of *x*-components and *y*-components

	n	Experiment 1 52		Experiment 2 40		Experiment 3 45		Experiment 4 60	
		x	*y*	*x*	*y*	*x*	*y*	*x*	*y*
Rectangular components of the displacement (mean±s.d.)		0.00±8.42	−2.04±9.38	1.24±11.29	−1.63±11.05	−1.29±16.18	1.63±21.41	8.01±16.66	0.02±15.30
Test value		0.005	1.553	0.687	0.924	0.528	0.504	3.69	0.008
Probability		>0.9	>0.1	>0.4	>0.3	>0.6	>0.6	<0.001	>0.9

Two-tailed one-sample Student's *t*-test to evaluate the mean values of the *x*-components (components along the tractive force; see Fig. 2b) and those of the *y*-components (components perpendicular to the tractive force) of the plots in Fig. 4. Note that only a mean value of the *x*-components in Exp. 4 (Fig. 4d) significantly shifts to the plus-side of the *x*-axis (P<0.1%).

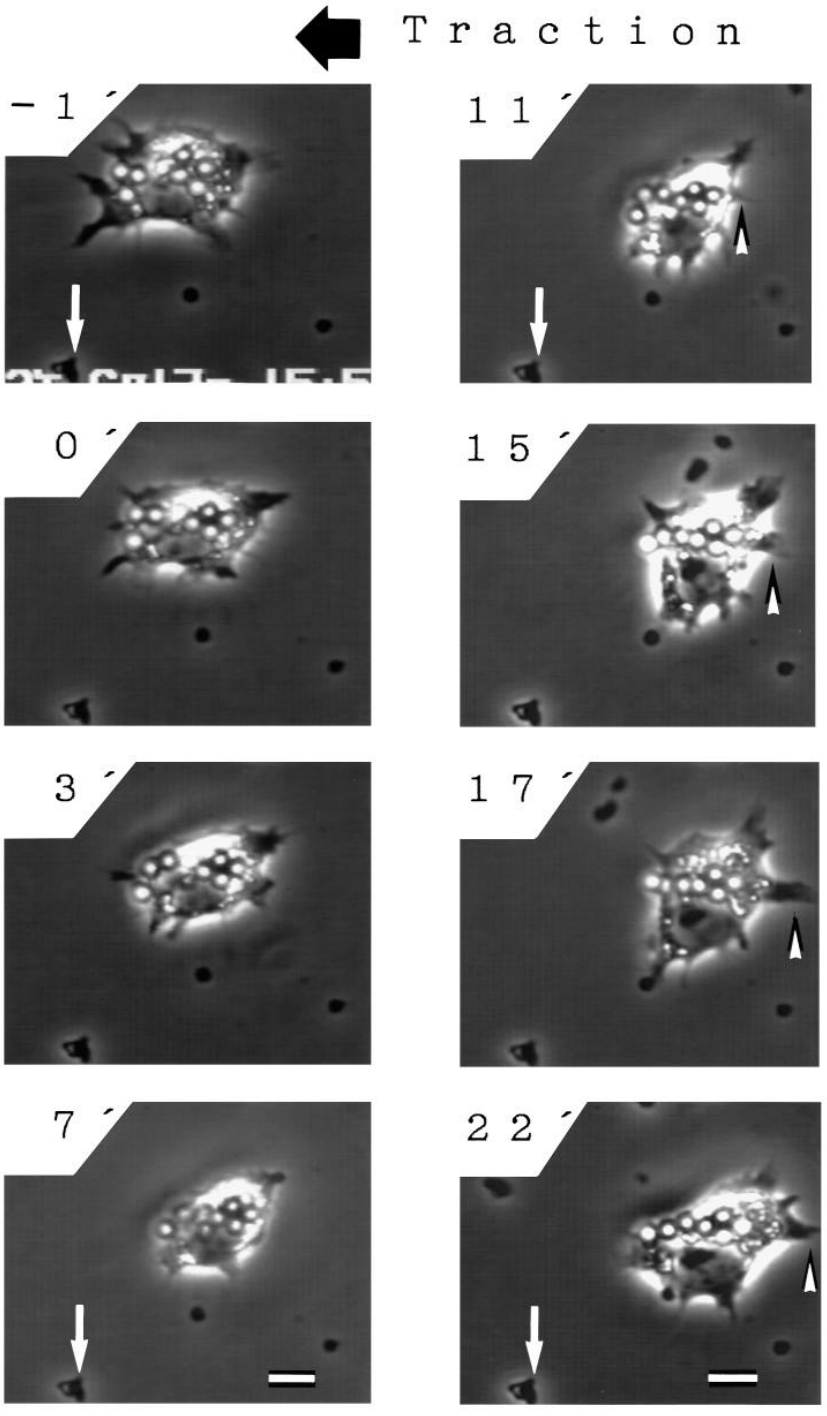

Fig. 8. Series of phase contrast micrographs of a mesodermal cell to show the organization of the magnetic beads adhered to the cell in the magnetic field. The time when the magnetic force was applied was shown with 0 time. Black arrow indicates the direction of the tractive force. White arrows indicate a plasma clot as a fixed point in the fields. Numbers in the frames show the passage of time in minutes after the onset of traction. Note that a cluster of beads change their positions for the magnet within several minutes and are beginning to line up along the magnetic field at the rear of the cell. Note also that a tongue-like leading lamella is projected in a direction precisely counter to the traction on the line through the tandem beads (arrowheads). Bars, 10 μm.

30 minutes, which was not observed in the control cases free of traction (Fig. 10a). Some of the cells in Exp. 4 did not locomote straight away from the tractive force, but vibrated forward and backward reciprocally along the line of magnetic force (Fig. 10b).

The scattergram in Fig. 4d shows that cell locomotion was biased in the direction counter to the force of traction. With parametric Hotelling's T^2-test at the 0.1% significance level or with the non-parametric alternative at 1%, it was judged that the distribution of the plots in Fig. 4d deviated from the origin to a direction (Tables 1 and 2). The angle held between the mean vector of the plots (corresponding to the center of the ellipse) and plus vector on the *x*-axis was +0.1° anti-clockwise (Table 1). The position of the center of the confidence ellipse ((8.01, 0.02) (μm); Fig. 5; Table 1) also indicates that the cells which were pulled in parallel with the substratum tended to move against the mechanical force. In support of this, both one-sample and two-sample *t*-tests (Welch's method) to the *x*-components (i.e. components parallel to the line of magnetic force) proved that the cells in Exp. 4 significantly preferred to move in a direction opposite to the traction (Tables 4 and 5). Average *x*-migration in Exp. 1 is 0.00±8.42 (μm), whereas in Exp. 4, it is 8.01±16.66 (μm) (mean ± s.d.). The test value of

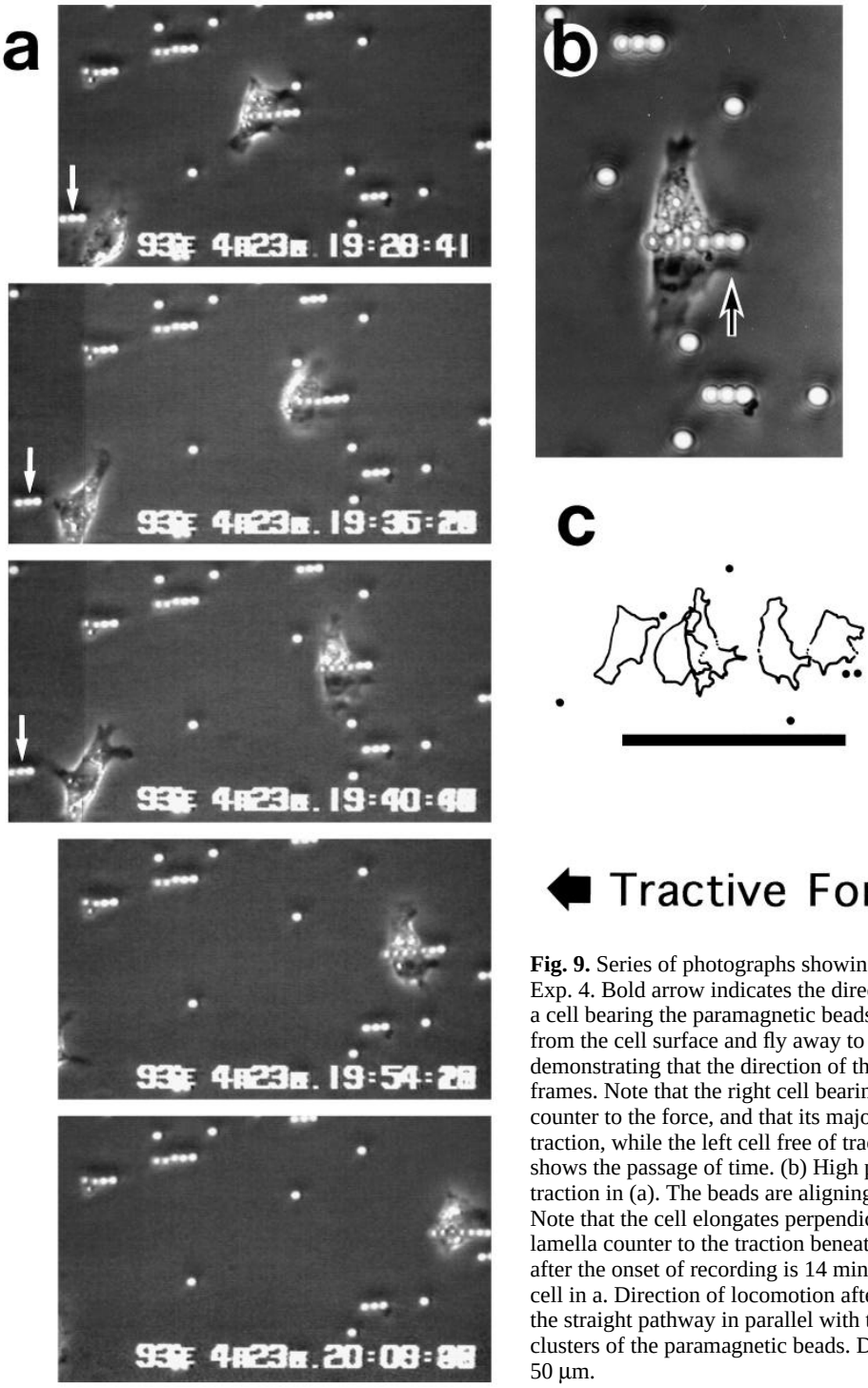

Fig. 9. Series of photographs showing the typical 'escape behavior from traction' in Exp. 4. Bold arrow indicates the direction of the traction. (a) Time-lapse recording of a cell bearing the paramagnetic beads under the traction. Beads on the left cell detach from the cell surface and fly away to the left in 5 minutes (white arrows), demonstrating that the direction of the magnet is actually for the left side of the frames. Note that the right cell bearing 6 beads continues to locomote in a direction counter to the force, and that its major axis usually orients perpendicular to the traction, while the left cell free of traction moves randomly. Number in each frame shows the passage of time. (b) High power view of the mesodermal cell under the traction in (a). The beads are aligning precisely along the line of the magnetic force. Note that the cell elongates perpendicular to the traction and protrudes a leading lamella counter to the traction beneath the line of the beads (arrow). Passage of time after the onset of recording is 14 minutes. (c) Trace of the outlines of the mesodermal cell in a. Direction of locomotion after 30 minutes was +5°. Note that the cell takes the straight pathway in parallel with the line of magnetic force visualized by the clusters of the paramagnetic beads. Dots indicate the fixed points on tracing. Bar, 50 μm.

Welch's *t*-test is 3.24 and the corresponding probability about the difference of the two *x*-migrations is less than 1%. On the other hand, y-migration between Exp. 1 and Exp. 4 was judged not to be different ($P>0.3$).

Distance of locomotion of the cells in Exp. 4 was proved to be longer than that of the cells in Exp. 1 or in Exp. 2, and almost the same as that of the cells in Exp. 3 (Table 3). In accordance with this, distance between the origin and the gravity of the plots in Exp. 4 was about four times longer than the others (Fig. 5; Table 1).

DISCUSSION

Mechanical force and the leading lamella

Chick gastrula mesodermal cells loading several paramagnetic beads and being pulled by the mechanical force in the magnetic field were observed to move against the traction. This was statistically confirmed with Hotelling's parametric T^2-test ($P<0.1\%$) or non-parametric alternative ($P<1\%$), which were developed to detect a significant deviation to a direction in a scattergram. Position of the center of the confidence ellipse in

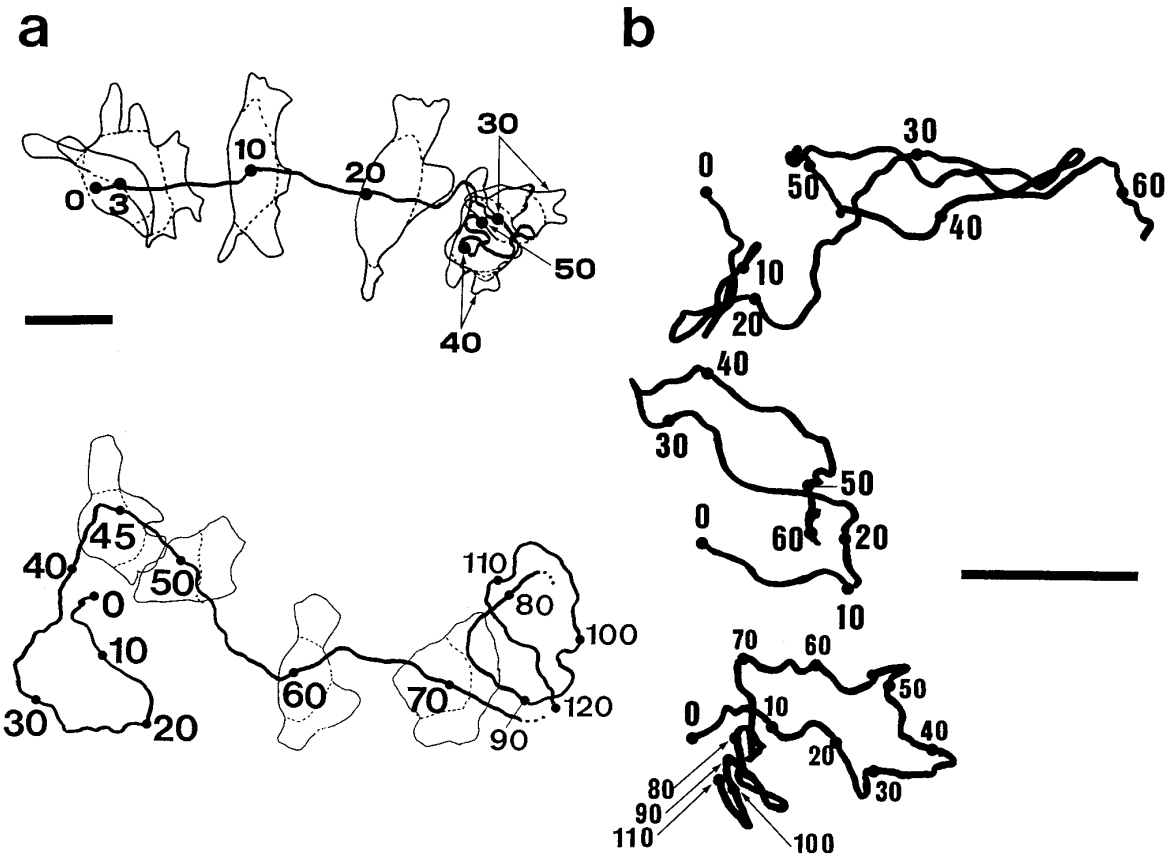

Fig. 10. Trajectories of the beads loaded on the mesodermal cells in the magnetic field as one of the parameters of cell movement. Each drawing represents a pathway of one bead. The cell took a similar route to the track of the bead, because the beads were located within the cell figure macroscopically. Numbers near the closed circles on the lines show the passage of time in minutes from the onset of recording. Bars, 10 μm. (a) Typical movement under traction. Note that a phase of straight locomotion in 'escape from traction' and a phase of random walk lacking directionality are recognized in these two samples, but in 'escape from traction' the beads traveled farther. In both cases, wandering locomotion occurred when the cells were blebbing. (b) Another type of typical movement showing reciprocal motion of a bead along the direction of the tractive force. In these three cases, the cells did not locomote straightaway from the magnet, but they vibrated reciprocally. Secondary reaction against the 'escape from traction' may have occurred in these cases.

Fig. 4d also assured us that the direction of locomotion was biased in a direction away from the magnet, that is, against the tractive force. One-sample *t*-test ($P<0.1\%$) and two-sample Welch's *t*-test ($P<1\%$) proved this. Thus we found a novel behavioral property of the migratory cells by pulling them in parallel with the migratory plane.

Then, which parts of the cell were most stretched by the force? Part of the paramagnetic beads attached to the cells were phagocytosed in the cells even when they were stressed by the magnetic field (Fig. 7), and the magnetic force lead the other beads, those simply anchored to the cell surface, to shift their position to the side facing the magnet (Fig. 8). On the other hand, at the most peripheral edges, focal contacts of the mesodermal cells to the substratum were formed (Fig. 3). In this condition, when a cluster of the beads at the cell center were pulled by the magnetic force, the leading lamella whose edge happened to be advancing against the tractive force must have been most effectively stretched. In Exp. 4, long tongue-like leading lamella was often formed in alignment with the traction (Figs 8 and 10).

Based on these results, we concluded that advancement of a

Table 5. Two-sample *t*-test for the difference of the average values (Welch's method)

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
x-components				
Experiment 1		0.57 (ϕ=70) $P>0.5$	0.48 (ϕ=64) $P>0.6$	3.24 (ϕ=90) $P<0.01$
Experiment 2			0.84 (ϕ=79) $P>0.4$	2.40 (ϕ=98) $P<0.02$
Experiment 3				2.87 (ϕ=97) $P<0.01$
y-components				
Experiment 1		0.19 (ϕ=76) $P>0.8$	1.06 (ϕ=59) $P>0.2$	0.86 (ϕ=100) $P>0.3$
Experiment 2			0.89 (ϕ=68) $P>0.3$	0.62 (ϕ=97) $P>0.5$
Experiment 3				0.43 (ϕ=76) $P>0.6$

Two-sample *t*-test (Welch's method) to search the significant differences between the mean values of the coordinates in Table 4. Note that only the *x*-components of the plots in Exp. 4 significantly shifts to the plus-side of *x*-axis as compared with the other ($P<1\%$), and that null hypothesis is established in all the combinations of *y*-components.

leading edge could be activated when the leading lamella is stretched longitudinally by a sufficiently strong mechanical force, and this leads the cells to locomote in a direction away from the force. Previous investigations support the above idea. Formation of a long cellular process was induced by local application of high frequency electric field by causing mechanical stress to the charged plasma membrane (Margolis and Povov, 1988; Margolis, 1991). By applying mechanical stimulation with a glass needle, polymorphonuclear leucocytes or keratinocytes extended a podium toward the needle (Korohoda et al., 1992). These mean that growth of the protrusion is closely related to the mechanical stress at the point. Advancement of the leading edges of corneal epithelia was enhanced along the strain by applying tensile stress parallel to the substratum (Takeuchi, 1979). Coincidentally, mechanical stress along the substratum exerted to an epithelium lead their microfilaments to align in parallel with the tension, and at the same time suppress the protrusive activity in the direction perpendicular to the axis of tension (Wong et al., 1983; Korega, 1986).

In preliminary studies, we tried to pull the mesenchymal cells excised from dermal placodes of 7 day chick embryos or those from limb buds of 3 day chick embryos in the same way as in Exp. 4. These cells took on typical fibroblastic forms even when more than 10 beads were attached to the cell surface, but no change of behavior occurred. We think tension inside the elastic cell body or adhesion to the substratum was so strong in these cases that the artificial extrinsic force of traction failed to produce enough strain to establish its new orientation inside the cells.

Traction strength sufficient (but not excessive) to evoke 'escape behavior from traction'

Adhesion plaques beneath the leading lamella are known to be terminated with F-actin bundles running through the lamella (Izzard and Lochner, 1976). We suppose that the contractile microfilament system in the leading lamella (Isenberg et al., 1976) plays a decisive role in the traction-induced orientation of cell locomotion. The microfilament system changes its amount (Pender and Mcculloch, 1991) and visco-elastic nature (Sato et al., 1987) in response to the mechanical stress. Oiwa et al. (1990) placed a polystyrene bead coated by myosin heads on a polarized F-actin bundle and applied a centrifugal force to the bead in the direction of the bead movement. Interestingly, speed of the movement slowed down by increasing the loads, and then recovered towards the velocity without centrifugation. This observation suggests that sliding interaction between F-actin and myosin molecules was controlled by the mechanical stress applied to the system. Coincidentally, Yanagida et al. (1985) reported that sliding distance between a myosin head and an actin filament for one ATP hydrolysis cycle is not constant, and they proposed that the mechanical load applied to their system modify the sliding distance and control the power of contraction. Recently, Wang et al. (1993) reported that integrins act as mechanoreceptors and transmit mechanical signals to the cytoskeleton.

The power of traction in our system (approximately 10^{-10} N per cell; see Materials and Methods) was 100 times stronger than the force produced by a single actin filament interacting with several myosin molecules (Ishijima et al., 1991), and about 10-100 times weaker than the centrifugal force capable of detaching fibroblasts from the substratum coated with FN (Lotz et al., 1989; Truskey and Pirone, 1990). The force of traction in our system should have been of sufficient strength to evoke the 'escape behavior from traction'. In preliminary studies, we used carbonyl iron powder suspended in PBS supplemented with 10% FBS instead of the paramagnetic beads, and added the tensile stress to the mesodermal cells with the same neodymium magnet. In this condition, the strain was so strong as to detach the isolated mesodermal cells from the substratum and to strip off the mesodermal cell sheets. It was too strong.

Implication of our result on the crawling movement

Abercrombie (1970) first described the crawling movement of tissue fibroblasts. The movement consisted of two phases: continuous extension of the leading lamellae; and succeeding quick contraction caused by the detachment at one site of the leading edges. In this report, it is strongly suggested that traction parallel to the substratum of sufficient strength activates the advancement of the leading lamella, and this should be involved in the mechanism of the crawling movement. That is, the intrinsic tensile stress accompanied by the extension of a leading lamella accelerates the advancement of its leading edge, which thus forms a positive feedback system until detachment of an edge at one site occurs.

Implication of our result to the expansion of a mesodermal cell sheet in vivo

Chick gastrula mesodermal cells migrate as a densely packed cell mass on the basal surface of the epiblast (Vakaet, 1970; Takeuchi, 1984; Duband et al., 1988; Hatta and Takeichi, 1986; Duband and Thiery, 1990; see Harrisson et al. 1988). Direction of their migration is known to be guided by the fibrous extracellular matrix rich in fibronectin aligned on the surface (Critchley et al., 1979; Boucaut and Darribère, 1983; Toyoizumi and Takeuchi, 1992) like the migration of frog gastrula mesodermal cells on the blastoroof (Nakatsuji and Johnson, 1984; see Johnson et al. 1990). Expansion of the mesodermal cell layer proceeds continuously (Vakaet, 1970).

Why do the mesodermal cells migrate unidirectionally in vivo? One of the most acceptable explanations is that contact inhibition stops the movement toward cell condensation. However, this idea appears not to be so good to explain the tandem locomotion of the large part of the mesodermal cells while adhering to one another (Winklbauer, 1990; Toyoizumi et al., 1991). If the above 'traction-activated advancement' hypothesis is effective also in vivo, it provides a new simple explanation to the mesodermal layer formation as follows: leading lamella of a marginal cell of the mesodermal cell layer protruded into the free space must happen to be pulled back by the cohesive power to make cell aggregation. If this force induces the 'traction-triggered locomotion' of the marginal cell, it will facilitate the expansion of the mesodermal cell layer. During this procedure, the motion should be guided by the alignment of fibrous ECM. A similar explanation may be applied to the spreading of the epithelial sheet (see Middleton, 1982).

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