INDUCTION OF SPREADING DURING FIBROBLAST MOVEMENT

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ABSTRACT

This paper describes the phenomenon of retraction-induced spreading of embryonic chick heart fibroblasts moving in culture. Measurable criteria of cell spreading (increase in area of the spreading lamella, and total spread area of the cell) are found to change predictably with retraction of a portion of the cell margin. Ruffling activity was found to increase. The leading lamella of a spread fibroblast ordinarily advances slowly, with an average area increase of ~21 μ^2m/min. A 10- to 30-fold increase in spreading occurs within 8 s after onset of retraction at the trailing edge and then decreases slightly so that by 1 min the increase in spreading is five to tenfold. During this period, there is a linear relationship between area increase at the leading edge and area decrease at the trailing edge. During the next 10–15 min, spreading gradually decreases to normal. Although the relationship between area spreading and area retracting of fibroblasts at different phases of movement is not significantly linear, it is highly correlated (Table II). These results suggest that the rate of fibroblast spreading may be inversely related to the degree of spreading of the cell as a whole.

KEY WORDS control of spreading · cell movement · fibroblast · cell motility · cell surface arrangement · cancer spreading

Cellular movement has been shown to play an active role in morphogenesis and in the spread of cancer (see reference 14 for review), yet the question of how cells regulate the speed and directionality of their movement is little understood. Based on the observation that fibroblast-like cells in vitro spread on plane substrata to form a varying number of flattened lamellae, it has been proposed that translocation of the cell as a whole results from a "tug of war" among these lamellae (1, 12, 15, 16). If this is so, it suggests that detachment of part of the cell surface from the substratum, with the accompanying abrupt retraction, would immediately increase spreading elsewhere on the cell surface.

To test this hypothesis, and thus shed light on the control of cell movement, I have observed the behavior of embryonic chick heart fibroblasts, moving on a plane glass substratum in culture, when part of the cell is detached from the substratum and retracts. Detachment and retraction and their effects on spreading were observed under two circumstances. (*a*) Normal detachment and retraction of the long, tapering trailing portion of the cell; and (*b*) artificial detachment of this trailing edge with a microneedle controlled by a micromanipulator. In all cases, detachment and retraction were followed by an immediate spurt in protrusive activity at the current leading edge of the cell. I have termed this phenomenon retraction induced spreading.

This paper will describe these results, both qualitatively and quantitatively, and discuss a possible mechanism. Part of the results of this research was presented at the 18th annual meeting of the American Society for Cell biology (8).

MATERIALS AND METHODS

Suspensions of embryonic chick heart fibroblasts were derived from the ventricle of 7- to 8-d embryos and cell culture were obtained according to the method of Armstrong and Lackie (4).

The effect of both naturally and artificially induced retraction of the trailing edge on cell spreading was

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Volume 81 June 1979 684-691

recorded with time-lapse cinemicrography. Artificial detachment of the trailing edge of a moving fibroblast was performed with glass microneedles, made by a vertical pipette puller (Model 700c, David Kopf Instruments, Tajunga, Calif.), mounted in a Leitz micromanipulator (E. Leitz, Inc., Rockleigh, N. J.). Needles which tapered sharply were found to be most useful, as more gradually tapering needles were too flexible to detach cells. To obtain a better optical result and maintain the medium in constant condition, a No. $1^1/_2$ glass coverslip was sealed with heat and paraffin to the underside of a hole 16 mm in diameter cut through the bottom of a 60-mm plastic petri dish. Fibroblasts were cultured directly on the coverslip in the bottom of the petri dish and the dish was flooded with 4 ml of medium. This micromanipulation chamber was placed on the stage of a Zeiss Universal microscope (Carl Zeiss, Inc., N. Y.) in air and a ×40 phase water immersible objective and a microneedle were immersed into the medium. Mineral oil saturated with medium and 5% CO_2-air mixture was placed over the medium during manipulation to prevent evaporation. The microneedle was inserted at such an angle that the very tip contacted the substratum and bent parallel to it for a very short distance. A wiping motion at the tip gradually advanced it from the extreme margin of the cell toward the cell body until the trailing edge of the cell was detached and retracted. Fibroblasts with a taut, phase dark trailing portion (Fig. 1), indicating that they were about to undergo natural retraction, were chosen. All experiments were performed in a 37°C room.

Time-lapse films were made with either a Bolex 16 mm camera with a Sage intervalometer or an Arriflex 16 mm camera and intervalometer. 4-s intervals were usually used for filming and 1 to 2 s for recording micromanipulation, with an exposure in each case of 0.5 s. The film used was Kodak plus-X Reversal, Type 7276, processed commercially. Films were projected for analysis with either a photo-optical Data Analyzer (L-W International, Woodland Hills, Calif.) or a Vanguard Motion Analyzer (Vanguard Instrument Co., Melville, N. Y.).

Tracings of cell outlines were made from projected images of 16 mm high resolution Nomarski differential interference or phase contrast cine films. For each set of data, tracings were made either every minute of real time for rapid movement or every 3 min of real time for slow movement. The total spread (outlined) area enclosed within the traced cell outline was then measured using an electronic planimeter (Numonics Graphics Calculator, Model 1224, Numonics Corporation, Penn.), and this value (in mm^2) was converted into actual area in μm^2 by dividing by the magnification and projection factors of the film. To measure the increase of area at the leading edges and the decrease of area at the trailing edge between two sequential times, two tracings of cell outlines were superimposed (Fig. 2), and areas enclosed in two leading edges and in two trailing edges were measured separately with the planimeter.

To determine the proportion of the cell periphery that was ruffling at a given time, I used a modification of the margin sampling method of Bell (5). A polar grid, composed of 36 radii of a circle, intersecting at a common center and separated by even spaces of 10° of arc, was drawn on the screen. The film was projected so that the grid was centered on the cell body but was otherwise randomly oriented. The point where each of the 36 radii intersected a ruffle was then scored at intervals equivalent to 1 min of real time. In this way, one could determine whether ruffling activity had changed. The number of intersected points converted to a percentage of the total number of sampling lines was taken as a measure of the percentage of the entire cell margin that was ruffling.

RESULTS

Induced Spreading

As an embryonic chick heart fibroblast continues to move in one direction, the trailing portion of the cell becomes very extended and it finally detaches and retracts. The mode of movement of one of these cells is illustrated in prints of frames taken from a time-lapse film (Fig. 1). The main features of this movement are characteristic of many others observed under similar conditions.

To quantify the possible competitive relationship of the various regions of the cell during locomotion, planimetric measurements were made of the decrease in area at the trailing edge and of the increase in area at the leading edge (Fig. 2). Since both speed of spreading and speed of retraction in a moving fibroblast can be measured simultaneously with the planimetric method (Fig. 2), this method provides accurate quantitative information on the relation between retraction and spreading. Fig. 3 shows the time-course relationships among spreading at the leading edge, retraction at the trailing edge, total spread area, and ruffling activity (see Materials and Methods) of a fibroblast undergoing natural detachment of the trailing edge, which is representative of seven others analyzed in the same way. Retraction of the trailing edge was also induced artificially by detaching it from the substratum with a microneedle (see Materials and Methods) to determine if the same relationships (Fig. 4 and six other cases studied) occur whenever there is retraction at the trailing edge, regardless of the cause.

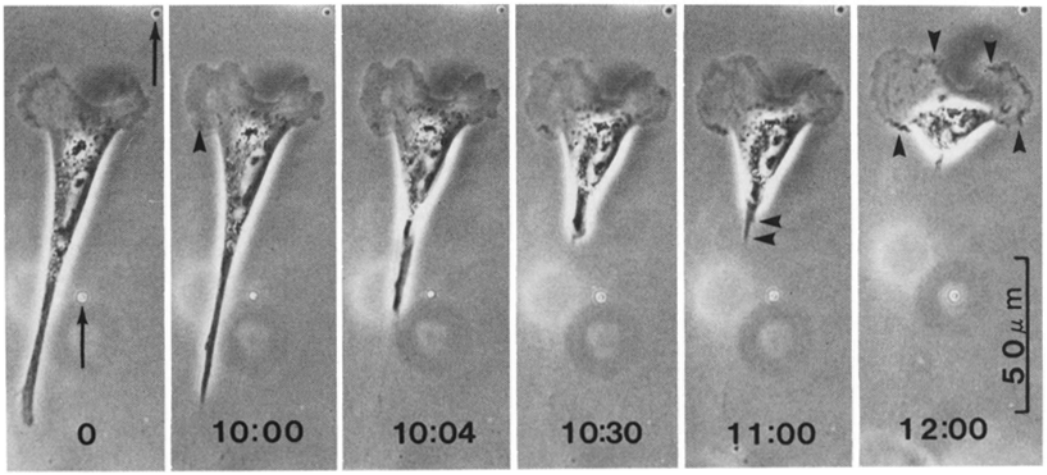

FIGURE 1 Abrupt retraction of the trailing edge of an embryonic chick heart fibroblast followed by an increased rate of spreading of the leading lamella. The phase-contrast micrographs are prints of frames of a time-lapse film. Numbers refer to minutes and seconds after beginning of the observations. Debris adherent to the substratum (arrows) are used as fixed reference points. (*0*) A highly polarized fibroblast with two distinct leading lamellae toward the top of the frame. A third and much smaller lamella is found at the trailing end of the cell. The two leading lamellae have broad thin lamellipodia at their extreme edges (3). The contour of the margins at the sides of the cells between the leading lamellae and the trailing lamella is concave, indicating tension or absence of adhesion to the substratum (13). (*10:00*) The cell has progressed forward slightly and is about to retract its trailing edge, which is now drawn to a point, presumably where the firmest adhesions are located. Part of the cytoplasm of the trailing portion of the cell has moved toward the main, forward part of the cell body. With this, the trailing lamella has become highly refractive, as indicated by its phase-dark appearance. The two leading lamellae advanced to a small extent, but have little ruffling activity (arrowhead). (*10:04*) ~3 s after the detachment of the trailing edge. The trailing edge is in the process of snapping back rapidly toward the cell body. Increased spreading at the leading edge is already apparent, particularly of the lamella on the left, and has been confirmed by planimetric measurements (see Fig. 3). (*10:30*) The cell is now at the slow phase of retraction (7) and the main cell body of the cell is now visibly shorter and wider. The increase in speed of spreading at the leading edges is now even more obvious. (*11:00*) The trailing portion of the cell is now almost completely retracted and is being gradually absorbed into the main cell body. A new taut trailing edge (double arrowhead) is now evident, indicating a second firm adhesion site. The leading lamellae have now spread about ten times as much as they do ordinarily in this period of time. (*12:00*) Vigorous ruffling (arrowheads) has appeared at the leading edge and the new trailing edge has detached and is retracting abruptly. × 400.

Artificial detachment at the trailing edge of a fibroblast causes an abrupt retraction, which does not appear to differ from that which occurs naturally (7). Moreover, the leading edge always suddenly increases its rate of spreading, just subsequent to this retraction, as shown in Fig. 3*a* and Fig. 4*a* (84 of 84 cases). The time-course of the decrease in area resulting from retraction of the trailing edge is closely associated with the subsequent increase in area of the leading edge. A most interesting result of these measurements is the revelation that the total spread area of a fibroblast generally remains constant when the cell is spreading at a constant rate (Figs. 3*b* and 4*b*). It declines somewhat and fluctuates when the trailing edge abruptly retracts and spreading at the leading edge suddenly increases, indicating that the decrease in area accompanying retraction at the rear end is not immediately compensated for by the surge in spreading at the leading edge. However, it is quickly restored to the value before retraction of the trailing edge, ~10 min after detachment (Figs. 3*b* and 4*b*), by increased protrusion at the leading edge. Ruffling activity also increases in response to detachment at the trailing edge and reaches its peak 3 min after detachment (Figs. 3*c* and 4*c*). All these responses at the leading edge to the artificially produced retraction of the trailing edge closely resemble those following natural retraction, ex-

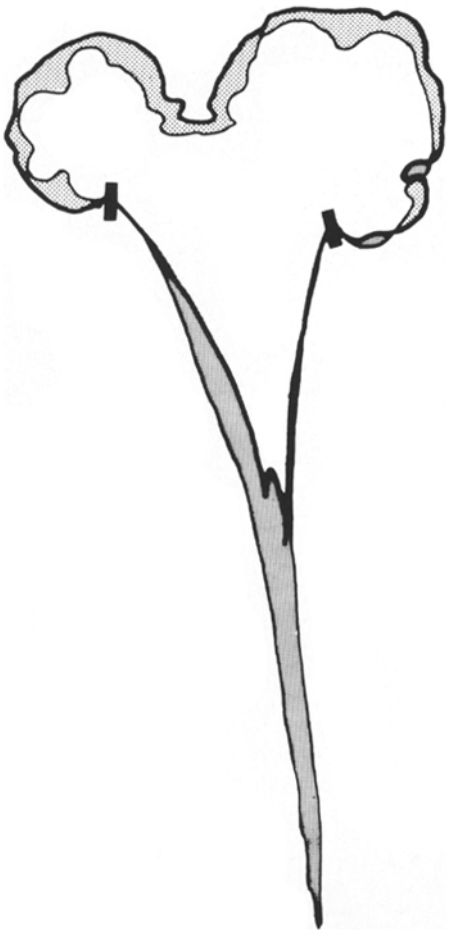

FIGURE 2 Planimetric measurement of the directional spreading of a fibroblast as a whole. Two superimposed sequential cell tracings illustrate the method used to determine the decrease in area upon retraction of the trailing edge and the subsequent increase in area at the leading edge of the same cell. Area decrease at the trailing edge and area increase at the leading edge are indicated by light stippling. Since the cell margin just behind the leading lamella does not change position, it was used as a stable region, indicated by bars, to superimpose the two tracings of the cell, before retraction of the trailing edge and after accelerated protrusion of the leading edge. 1 min elapsed between retraction and protrusion in this particular case.

cept that occurring the minute immediately preceding detachment (see below).

Analysis of the Rate of Movement

The speed with which the trailing edge of a fibroblast retracts and the leading edge subsequently surges ahead is striking, especially in time-lapse films, in which both processes seem almost simultaneous, due to the 50-fold acceleration in time. Even when observed in real time, however, spreading of the leading edge of a fibroblast is observed to accelerate at the same time as the trailing edge retracts. In a word, this is truly a rapid phenomenon, especially when compared with its almost imperceptibly slow rate of normal spreading.

In this section, I describe the results of statistical analysis (Tables I and II). A spread fibroblast generally moves slowly, with an increase in area at the leading edge and a decrease in area at the trailing edge of ~21 μm^2/min (Tables I and II, −20 to −1 min), or 0.35 μm^2/s. With abrupt detachment at the trailing edge, naturally or

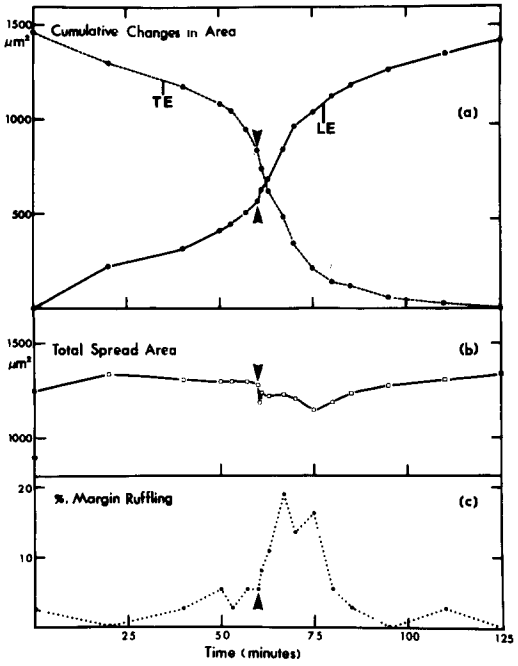

FIGURE 3 Single cell analysis of the time-course relationships among spreading at the leading edge, retraction at the trailing edge, total spread area, and ruffling activity at the leading edge of a fibroblast undergoing abrupt retraction of the trailing edge. (*a*) These two curves show the cumulative areas of spreading at the leading edge and of retraction at the trailing edge, using the planimetric method illustrated in Fig. 2. Detachment at the trailing edge is indicated by an arrowhead. (*b*) Total spread area of the cell at the sequential time is measured simply by tracing the whole cell margin with the planimeter. (*c*) Changes in percentage of the margin ruffling, measured by the margin sampling method (see Materials and Methods).

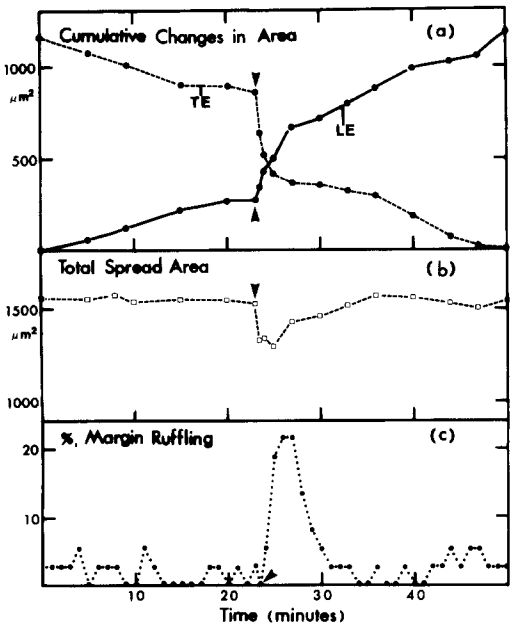

FIGURE 4 Single cell analysis of a spread fibroblast artificially induced to spread with micromanipulation. The trailing edge of a spread fibroblast is artificially detached by a glass microneedle at the time indicated by an arrowhead. Methods used to obtain the data are described in Fig. 3.

artificially with a microneedle, retraction at the trailing portion of the cell occurs very rapidly, indeed within the first 8-s after detachment, at an average speed of 30 $\mu m^2/s$ (8 measurements). A burst of spreading at the leading edge also occurs within this first 8-s period, and varies in area increase from 132 μm^2/min (2.2 $\mu m^2/s$) to 618 μm^2/min (10.3 $\mu m^2/s$), with average of ~240 μm^2/min (4.0 $\mu m^2/s$) (8 measurements). This represents an increase in area at the leading edge up to 30-fold.

Since the first, fast phase of retraction at the trailing edge is always followed by a second, slow phase (7), with the result that the whole retraction process takes an average of a full minute, changes in area of a group of seven cells in response to natural detachment of their trailing edge were measured at 1 min intervals for 5 min after detachment (Table I). Changes in area 1 min before and after artificial detachment of seven cells are also presented for comparison. Although area increase and area decrease both subside after the initial 8 s burst, the average increase in area 1 min after detachment is still sixfold, and the decrease in area tenfold, the normal speed (Table I).

When the areas of spreading and the areas of retraction within the first minute after detachment of the trailing edge of 16 cells were plotted together, in a linear regression (Fig. 5), the points appeared to be linear, indicating a linear

TABLE I

Rate of Increase in Area of the Leading Portion and Decrease in Area of the Trailing Portion of Fibroblasts, with Special Reference to Abrupt Retraction at the Trailing Edge

Time*		Rate‡
min		*μm^2/min*
−20 → −1	A§	21 ± 10 (14)
	B‖	20 ± 11 (14)
+10 → +30	A	22 ± 8 (14)
	B	22 ± 10 (14)
Natural detachment		
−1 → 0	A	35 ± 15 (7)
	B	65 ± 50 (7)
0 → +1	A	127 ± 51 (7)
	B	239 ± 77 (7)
+1 → +2	A	44 ± 46 (7)
	B	49 ± 43 (7)
+2 → +3	A	81 ± 40 (7)
	B	68 ± 44 (7)
+3 → +4	A	56 ± 58 (7)
	B	75 ± 66 (7)
+4 → +5	A	28 ± 33 (7)
	B	35 ± 49 (7)
Artificial detachment		
−1 → 0	A	19 ± 13 (7)
	B	16 ± 11 (7)
0 → +1	A	128 ± 47 (7)
	B	266 ± 118 (7)

Seven embryonic chick heart fibroblasts (1-2 d in culture) with natural detachment of their trailing edges and seven cells with artificial detachment of their trailing edges were examined over a period of 50 min. Speeds of spreading at the leading edge and of retraction at the trailing edge at a given time were measured with the planimetric method illustrated in Fig. 3. Since the limit of resolution of the light microscope is ~0.1 μm and since there are some errors generated by projecting and tracing cell outlines as well as by the planimetry, the areas measured are presented primarily with two significant figures.

* Time in min with reference to detachment at the trailing edge (0); −, mins before detachment; +, mins after detachment.

‡ Data are given ± SD (number of cases)

§ A = Rate of increase in area of the leading portion.

‖ B = Rate of decrease in area of the trailing portion.

relationship between spreading at the leading edge and retraction at the trailing edge. This agrees with the observation that the surge of spreading at the leading edge immediately after detachment of the trailing edge correlates directly with the size of the trailing portion retracted (Figs. 3*a* and 4*a*).

In the second minute after retraction of the trailing edge (Table I), area increase of the leading edge is still augmented, but less so. It also fluctuates greatly, and ruffling activity becomes significantly increased (Figs. 3*c* and 4*c*). It should be noted that measurements of induced spreading made at later times (Table I and also 0–10 min in Table II) have greater standard deviations. Since in some cells there are subsequent minor retractions of the trailing portion of the cell after the first abrupt retraction and since I have shown that major changes in speed of spreading of the leading edge are highly correlated with major retractions of the trailing edge (Fig. 5), it seems reasonable to suggest that the variations in spreading at later times are also correlated with variations in later retractions.

Rate of induced spreading gradually declines to normal by 10–15 min after the onset of retraction. The average speed of this retraction-induced spreading within the entire first 10-min period is 72 ± 57 μm^2/min (Table II) and represents a threefold increase in spreading over the normal rate. Since increased spreading at the leading edge always follows abrupt retraction of the trailing edge, regardless of the cause, it seems reasonable to conclude that it can be induced by retraction of a portion of the cell margin.

It should be noted that area increase of the leading edge and area decrease of the trailing portion of the cell both accelerate within the minute immediately preceding natural detachment of the trailing edge (Table I) (a change in area increase from 21 ± 10 μm^2/min to 35 ± 15 μm^2/min, $t = 2.666$, $df = 21$, $p = 1.450 \times 10^{-2}$ and area decrease from 20 ± 11 μm^2/min to 65 ± 50 μm^2/min, $t = 3.539$, $df = 21$, $p = 1.943 \times 10^{-3}$). In contrast, however, there is no significant difference in the speed of spread cells 1 min immediately before artificial detachment of the trailing edge (Table I) (spreading: $t = 0.306$, $df = 21$, $p = 0.763$; retraction: $t = 0.707$, $df = 21$, $p = 0.487$). This correlation of accelerated increase in area of the leading lamella with accelerated decrease in area of the trailing portion of the cell immediately preceding the detachment provides a control for the possibility that detachment by itself causes an increase in spreading, and provides additional evidence that a simple decrease in area in one part of the cell is responsible.

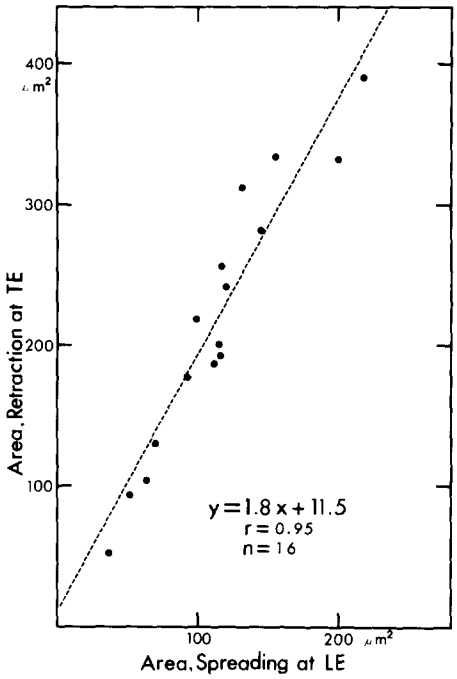

FIGURE 5 Correlation between spreading at the leading edge and retraction at the trailing edge of fibroblasts. The increase in area (x) at the leading edge (LE) and the decrease in area (y) at the trailing edge (TE) within the first minute after detachment of the trailing edge of a group of 16 cells were plotted together. The line drawn through the points was obtained by a linear regression using the method of least squares. The valve of correlation coefficiency (r) calculated is high, indicating a linear relationship between spreading and retraction.

DISCUSSION

This report shows that measurable criteria of fibroblast spreading (increase in spreading area of the leading lamella and ruffling activity) augment predictably with retraction of the taut trailing portion of the cell. This suggests that autonomous fluctuations in speeds of fibroblast spreading might always vary directly with the retraction-spreading cycle of the cell. The mitotic-intermitotic cycle of fibroblasts is a case in point (2, 6). Planimetric measurement of postmitotic spreading shows that rounded postmitotic daughter cells spread at a higher speed, similar

TABLE II

Correlation between the Rate of Increase in Area of the Leading Portion and Decrease in Area of the Trailing Portion of Fibroblasts at Different Phases of Movement, as Compared to the Same Phenomena after Abrupt Retraction of the Trailing Edge

						$y = \alpha + \beta x$				
Time	n	$\bar{x}$	S_x	$\bar{y}$	S_y	α	β	r	z	p
min		$\mu m^2/min$								
$-20 \rightarrow -1$	68	22	10	23	12	4	0.88	0.74	6.05	7.90×10^{-10}
$0 \rightarrow +10$	73	72	57	89	94	15	1.03	0.63	5.32	5.32×10^{-8}
$+11 \rightarrow +30$	68	30	15	29	12	11	0.58	0.69	5.66	7.51×10^{-9}

Time in min with reference to detachment at the trailing edge (time 0): −, min before detachment; +, min after detachment; n, number of measurements; $\bar{x}$, mean rate of increase in area of the leading portion; S_x, standard deviation of the x values; $\bar{y}$, mean rate of decrease in area of the trailing portion; S_y, standard deviation of the y values; $y = \alpha + \beta x$, the equation of linear regression; α, y intercept of the equation line; β, the slope of the equation line; r, coefficient of correlation; z, critical-ratio z-test value; p, probability level using Gaussian distribution. Since the limit of resolution of the light microscope is ~0.1 μm and since there are some errors generated by projecting and tracing cell outlines as well as by the planimetry, the areas measured are presented primarily with two significant figures. Movement of fibroblasts is arbitrarily divided into three phases: a spread state (−20 to −1 min before a detachment), a retraction-induced state (0–10 min after detachment), and a postinduction spread state (11–30 min after detachment). The slopes of the equation lines (β) obtained from linear regression for each phase of fibroblast movement vary, indicating different relationships between spreading and retraction at different phases. Much greater values of standard deviation (S_x and S_y) are found in the retraction-induced state (see text for detailed description). Since all three resulting probability levels obtained from Gaussian distribution are <0.01, the data are significant at the 0.01 level, i.e., there is a relationship between speeds of spreading and retraction of a fibroblast at the given times measured.

to that of abruptly retracted moving cells (8). A retracted state seems to favor spreading of fibroblasts.

Observations of the initial spreading of retracted cells (Figs. 1, 3, and 4) shows that the edges of all existing lamellae continue to spread outward until a fully spread state is reached. It is after this initial spreading that the various lamellae of the fibroblast appear to spread competitively, with a resulting "tug of war" among them (12, 16), the movement of the cell being always in the direction of the largest lamella (16). The question of interest is whether increased protrusive activity at the largest lamella depends on retraction at another margin or whether this dominant lamella generates sufficient force on its own to pull the rest of the cell along. The demonstration in the present study that increased spreading at one margin can be induced by detachment at another clearly favors the former idea. Moreover, this seems to be a general phenomenon. Weiss and Garber (16) remarked, for example, that a new margin comes into protrusive activity as one withdraws. And, also, when protrusive activity of the leading edge of a moving fibroblast is inhibited by contact with the edge of another cell, increased protrusive activity quickly develops elsewhere and forms a new leading edge (15). Erickson (9) recently reported that this increased protrusive activity in effect can turn or steer a BHK cell so that it aligns itself parallel to the cell it contacts.

The situation seems to be the same in the extension of neurites in culture. Artificial detachment of one part of a growth cone, or extending tip, with accompanying retraction, increases the protrusive activity of microspikes of the other part (17). Thus, one can "steer" the extension of a neurite in one direction or another at will. This has also been found to be true for fibroblasts; when one part of a leading lamella is detached, the other part shows increased spreading (8).

The fact that abrupt retraction at the trailing edge of a fibroblast is followed by a surge of spreading at the leading edge that is linearly related to retraction (Fig. 5) suggests that retraction of a portion of a fibroblast may make cell surface (and associated cytoplasmic material) available for renewed spreading elsewhere (see the hypothesis of Wolpert and Gingell [18]). This could occur by way of the folds and microvilli that form on the surface of a fibroblast immediately after retraction of the trailing edge (7). There is now much evidence that cell surface

conserved in folds, blebs, and microvilli may serve as a reserve for subsequent spreading (10, 11). This transient appearance of folds and microvilli upon retraction could also explain the transient reduction in total spread area that is evident immediately after retraction of the trailing edge in Figs. 3*b* and 4*b*. Since the surface area determinations on which these curves are based depended on planimetric measurements of cell outline, compression of the cell surface into folds and microvilli would show up as a loss in total area. In a fully spread cell, a limiting factor of cell spreading could be the amount of surface membrane and cortical cytoplasm available for forming protrusions, such as lamellipodia and microspikes. This constraint is temporarily relieved when the trailing portion of the cell detaches. During this time, the limiting factor becomes the rate of attachment of these surface protrusions to the substratum. Surface changes associated with retraction induced spreading and the significance of the phenomenon for the mechanism of fibroblast locomotion will be considered in detail in a subsequent communication.[1]

I am grateful to J. P. Trinkaus for his advice during this research and for his assistance in the preparation of this manuscript. I also wish to thank E. Davis, R. E. Keller, G. P. Radice, M. S. Shure, and N. K. Wessells for helpful discussions, and M. Trinkaus for editorial assistance.

This work represents part of a dissertation submitted to the Graduate School of Yale University in partial fulfillment of the requirements for the degree of Doctor of Philosophy and was supported by National Institutes of Health grants HD07137 and CA22451 to J. P. Trinkaus and by Yale University Fellowships to W.-T. Chen.

Received for publication 13 February 1979, and in revised form 15 March 1979.

[1] Chen, W.-T. Surface changes during retraction-induced spreading of fibroblasts. Manuscript in preparation.

Note Added in Proof: Graham Dunn informs me (personal communication) that he too has observed this phenomenon in moving chick heart fibroblasts (G. A. Dunn, 1979. Mechanisms of fibroblast locomotion. *In* B. S. C. B. Symposium on Cell Adhesion and Motility. Cambridge University Press, London. (In press.)

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