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TGFβ1 induces a cell-cycle-dependent increase in motility of epithelial cells

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SUMMARY

We have previously shown that addition of type 1 transforming growth factor-β (TGFβ1) to an exponentially growing population of mink lung CCl64 cells increases their average intermitotic time from 14.4 to 20.3 hours, predominantly by extending G_1 from 7.5 to 13.5 hours. Here we have used the DRIMAPS system (digitally recorded interference microscopy with automatic phase-shifting) for obtaining data on cellular mass distribution, cell motility and morphology. We found no significant change in the cells' rate of mass increase following TGFβ1 treatment, which implies that the treated cells attained a higher mass during their extended cell cycle and this was confirmed by direct measurement of cell size. However, the cells showed a dramatic motile response to treatment: TGFβ1-treated cells had a significantly higher time-averaged speed of 36.2 μm hour^{-1} compared to 14.5 μm hour^{-1} for the control cells. The time course of the response was gradual, reaching a maximum mean speed of 52.6 μm hour^{-1} after 15 hours exposure. We found that the gradual onset of the response was probably not due to a slow accumulation of a secondary factor but because cells were dividing throughout the experiment and most of the response to TGFβ1 occurred only after the first cell division in its presence. Thus, taking only those cells that had not yet divided, the time-averaged speed of treated cells (26.1 μm hour^{-1}) was only moderately higher than that of untreated cells (14.9 μm hour^{-1}) whereas, for those cells that had divided, the difference in speed between treated cells (45.1 μm hour^{-1}) and untreated cells (14.1 μm hour^{-1}) was much greater. Increased speed was a consequence of enhanced protrusion and retraction of the cell margin coupled with an increase in cell polarity. TGFβ1 also increased the mean spreading of the cells, measured as area-to-mass ratio, from 3.2 to 4.4 μm^2 pg^{-1}, and the intracellular mass distribution became more asymmetric. The observations indicate that a G_2 signal may be necessary to reach maximal motility in the presence of TGFβ1.

Key words: Quantitation of cell behaviour, Interference microscopy, Cell growth, Mink lung cell, Mv1Lu, DRIMAPS

INTRODUCTION

Type β transforming growth factors represent a family of polypeptides that modulate growth and differentiation of a wide variety of cell types (Massague, 1987; Roberts et al., 1985; Sporn et al., 1986). Three receptors for type 1 transforming growth factor-β (TGFβ1) have been characterized (denoted I, II and III) of which receptors I and II contain a serine/threonine protein kinase (Lin et al., 1992; Wang et al., 1991). Heterodimeric complex-formation of these receptors is necessary for downstream signalling (Wrana et al., 1992, 1994; Moustakas et al., 1993). A number of studies have indicated an effect of TGFβ1 on motility, for example chemotactic effects on fibroblasts, monocytes and hepatocytes (Postlethwaite et al., 1987; Wahl et al. 1987; Stolz and Michalopoulos, 1997). TGFβ1 has been studied extensively in mink lung epithelial cells since it was shown that these cells were growth inhibited by TGFβ1 and could be used to study its bioactivity (Cone et al., 1988). Apart from growth inhibition, a wide range of other responses to TGFβ1 in mink lung cells has since then been discovered, such as expression of the immediate early gene JunB (Pertovaara et al., 1989) and the induction of extracellular matrix components like fibronectin, thrombospondin and the plasminogen activator inhibitor-1 (Laiho et al., 1991).

In a previous study, using time-lapse video microscopy and flow cytometry, we have shown that the addition of TGFβ1 to mink lung epithelial cells results in an increase of the intermitotic time from 14.4 to 20.3 hours. This increase is predominantly due to an increased time spent in G_1 (Kramer et al., 1994). We also found that cell cycle inhibition was accompanied by a dramatic increase in cell motility.

In order to quantify the increased motility and to study further the cell morphological changes that accompany it, we have analysed the effects of TGFβ1 in mink lung epithelial cells using the DRIMAPS system (digitally recorded interference microscopy with automatic phase-shifting). This technique allowed us to obtain detailed information on cellular mass distribution, cell motility, spreading, shape and

polarity. We report that TGFβ1 induces a significant increase in motility in a cell-cycle dependent manner. Treated cells before division increased their speed to a maximum of about twice the basal level. Post-mitotic cells, however, showed a dramatic further increase in motility, reaching much higher speed levels. The increase in motility is due to increases in the rate of cellular protrusion and polarity and is accompanied by an enhanced spreading. Thus, although pre-mitotic mink lung cells show a significant response to TGFβ1, the maximal response is only developed in cells that have divided in its presence.

MATERIALS AND METHODS

Cell culture

Mink lung CCl64 (Mv1Lu) cells were kept in DMEM (Gibco BRL) containing L-glutamine supplemented with 10% foetal bovine serum (FBS, Gibco BRL), 100 units ml^{-1} penicillin and 1% streptomycin (Gibco BRL) at 37°C. Cells were passaged using two washes of calcium/magnesium free PBS (Imperial Laboratories) and a short treatment with 0.05% trypsin/Versene (Life Technologies Ltd) twice a week at 0.33 or 0.25×10^6 in 25 cm^2 flasks (Nunc). For analysis in the DRIMAPS system, cells were plated on acid-washed glass coverslips (22×22 mm) at various densities and left to adhere for four hours at 37°C in a humidified incubator containing 5% CO_2. Suitable cell numbers were selected, roughly 20 cells per observation field (530×360 μm). TGFβ1 (R&D Systems) was added to the experimental cultures at a concentration of 80 pM (2 ng/ml).

Coulter counter measurement of cell volume

Cells cultured for 38 hours in control medium or with additional TGFβ1 were released into suspension as described in the cell passaging procedure. Each cell suspension in culture medium was spun for 5 minutes at 700 ***g*** and the pelleted cells were resuspended in Isoton II (Coulter Electronics). The cell suspension was then measured in Coulter counter type ZBI with a 100 μm orifice (Coulter Electronics). Frequencies of cells in 256 cell-size channels were recorded and stored on computer hard disk. Cell size was estimated by calibration with latex beads of 14 μm diameter. Data were processed in Mathematica by polynomial regression and detection of the maxima.

DRIMAPS (digitally recorded interference microscopy with automatic phase-shifting) time-lapse system

Cells were mounted in a glass culture chamber (Dunn et al., 1997a) containing 0.33 ml of medium (±TGFβ1) and the chamber sealed in order to maintain a CO_2-buffered environment. The cells were examined using a Horn interference microscope (Leitz, Wetzlar, Germany) equipped with a ×20 double objective and kept at 37°C in a temperature-controlled room. Digital interference images were acquired from the microscope using a Pulnix TM765E camera and a Magic frame store (Matrox, Swindon, UK). A computer-controlled electronic shutter prevented illumination of the cells between exposures.

The principle of operation of the Horn interference microscope is that the illumination beam is split into two separate beams which pass through the double condenser and double objective. The two beams are recombined to interfere in the primary image plane after one of the beams, the object beam, has passed through the cell culture and the other beam, the reference beam, has passed through a reference chamber. The intensity level at each point in the interference image is a sinusoidal function of the optical path difference (OPD) between a light ray passing through the cell culture and another passing through the reference chamber. In the DRIMAPS system, phase-shifting allows the OPD to be calculated despite variations in the modulation of interference or in the intensity of illumination. To implement this, an additional phase shift between the two beams is introduced by means of a computer-operated optical wedge while four interference images are acquired. A set of three or more interference images acquired at different but known additional phase shifts, allows the OPD introduced by the cells to be calculated on a point-by-point basis (Dunn and Zicha, 1998). Since the OPD is directly related to the amount of non-aqueous cellular material in the light path, the resulting phase-calibrated images carry information about the dry mass density distribution in cells (Davies and Wilkins, 1952).

Recordings lasted for 20 or 32 hours and phase-calibrated images were taken at 1 minute lapse intervals. An image processing algorithm was then applied to the sequences of phase-calibrated images in order to remove fluctuations and distortions and to subtract a background image (Zicha and Dunn, 1995). Single cells in the resulting recordings were identified by interactive tracking and the final stage of the DRIMAPS procedure was to calculate various parameters of cell growth, movement and morphology from the identified cells. An example showing the motility of two cells calibrated for dry mass distribution is shown in Fig. 1.

Data analysis

Four control recordings giving 17,385 measurements for each parameter and six recordings with additional TGFβ1 giving 40,088 individual measurements were included in the main analysis. Seven aspects of cell behaviour are analysed in this study: rate of increase in cell mass, speed, spreading, asymmetry, cell shape, protrusion/retraction and polarity. Speed analysis was based on 1 minute displacements of the mass centroid (centre of gravity) of cells and expressed in μm $hour^{-1}$. Spreading was measured as ratio of total cell area to total cell mass and expressed in $\mu m^2\ pg^{-1}$. The

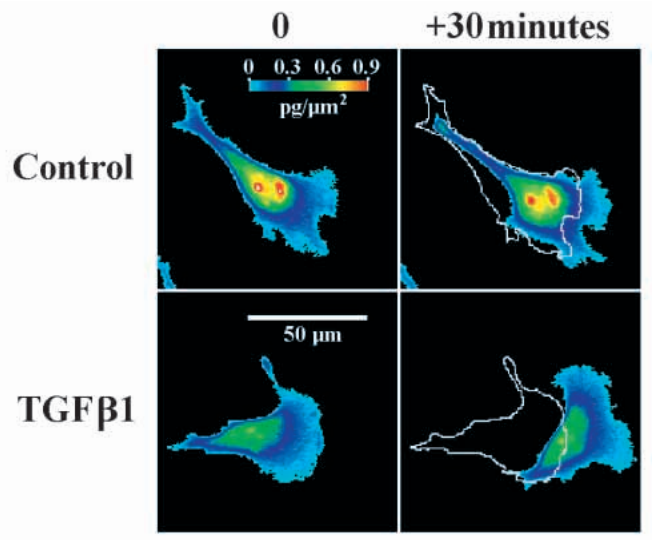

Fig. 1. Samples from DRIMAPS recordings demonstrating cell displacement over a period of 30 minutes. Outlines of cells at time 0 minutes are reproduced by white curves on images of cells taken 30 minutes later. During the 30 minute interval the TGFβ1-treated cell moved over a distance of 26 μm whereas the control cell only moved 7 μm. The pseudocolour scale represents mass density and it can be seen that this reaches a higher level in the control cell which indicates that the treated cell is more thinly spread. In the right-hand column, the protrusion regions are represented by those parts of the cells that lie outside the white outlines whereas retraction regions are the black areas within each white outline. In the actual analysis of data, much smaller intervals of 1 minute were used to calculate the areas of protrusion and retraction.

asymmetry of a cell was taken as the distance in μm between its mass centroid and its area centroid (centre of gravity based on cell outline). Cell shape was described by two parameters, developed by Dunn and Brown (1986), elongation and dispersion. Elongation represents the overall ratio between the length and width of a cell whereas dispersion describes how much the cell shape differs from an ellipse. Protrusion and retraction areas were calculated from comparison of the regions occupied by a cell in two frames 1 minute apart and were measured in μm^2. Protrusion was defined as the region newly occupied during the 1 minute interval and retraction was defined as the region from which the cell had withdrawn. In the right-hand column of Fig. 1, the protrusion regions are represented by those parts of the cells that lie outside the white outlines whereas retraction regions are the black areas within each white outline. In this example the frame interval is 30 minutes and so the areas of protrusion and retraction are greatly exaggerated for illustration. Polarity was measured as the distance in μm between the area centroids of the protrusion and retraction regions. A maximally polarized cell, with all the protrusion at the front and all the retraction at the rear, will have a polarity equal to the length of the cell whereas a minimally polarized cell, in which the protrusion and retraction regions are evenly interspersed around the margin, will have a polarity close to zero. The amount that a cell translocates during an interval depends on both the areas of protrusion and retraction and on their polarity.

Statistical significances of differences between control and TGFβ1-treated cells were calculated by analysis of variance (ANOVA) using an unbalanced nested model (Dunn et al., 1997a). Data were nested in five hierarchical levels: single measurement, continuous sequence of measurements, set of all continuous sequences belonging to a cell, set of cells belonging to a cell culture and the set of all cultures belonging to the control or treated groups. Time courses were generated by first averaging the 1 minute data belonging to a cell in 1 hour bins and then calculating averages over cells. Plots show mean values and 95% confidence intervals.

RESULTS

TGFβ1 does not alter the rate of increase in cell mass, thus creating larger cells

The rate of increase in mass of control cells, expressed as a percentage of total mass, was 0.0717% $minute^{-1}$ compared to 0.0705% $minute^{-1}$ for the TGFβ1-treated cells (Fig. 2A,B). These rates were not significantly different (ANOVA: $P>0.05$). Since we have found in a previous study that the cell cycle is approximately 6 hours longer for the treated cells (Kramer et al., 1994), this constant rate of growth in mass implies that the treated cells reach a larger size than the control cells during their extended cell cycle. We confirmed this prediction by analyzing cell diameter using a Coulter counter. After a 38 hour treatment with TGFβ1, cells had a mean diameter of 17.0 μm compared to 14.5 μm for untreated cells taken from the same batch and also cultured for 38 hours (Fig. 2C,D). This difference was highly significant in a *t*-test ($P<0.001$).

TGFβ1 induces a gradual increase in cell motility

In the analysis of cell motility, we first compared the speed of translocation of untreated mink lung cells with cells that had been treated with TGFβ1 from the beginning of recording. Fig. 3A shows that the mean speed of the untreated cells (black data points) did not vary greatly with time in culture and had a time-averaged value of 14.5 μm $hour^{-1}$. The treated cultures showed that TGFβ1 (green data points) induced a significantly higher time-averaged speed of 36.2 μm $hour^{-1}$ (ANOVA: $P<0.001$). This higher speed of the treated cells developed gradually with time in culture and reached a maximum of 52.6 μm $hour^{-1}$ after 15 hours. Although this increase in speed appears to show sudden steps, particularly at 9 hours, a polynomial curve of third order (black line) fitted by least squares to the data points lies within all the 95% confidence intervals. We therefore have no evidence to reject the hypothesis that the increase is a smooth process.

We next tested whether the slowness of the response could be explained by an indirect action of TGFβ1 such as a gradual release of some soluble chemokinetic factor following TGFβ1-treatment. If so, we would expect conditioned medium from TGFβ1-treated cultures to induce a much more rapid response. In four experiments, we took conditioned medium from cultures that had been incubated with TGFβ1 for 16 hours and added this to cultures of untreated cells (Fig. 3B). The onset of

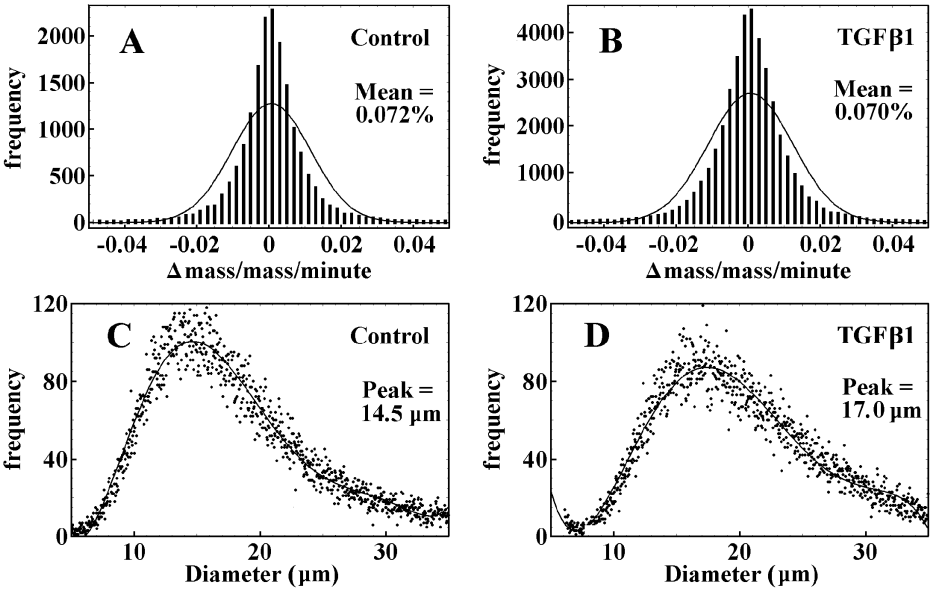

Fig. 2. (A and B) Histograms of relative cellular mass increase overlaid by Gaussian distribution of the same mean and deviation for control and TGFβ1-treated cells, respectively. Note that there is no significant difference in the mean rate of mass increase between the two. (C and D) Distributions of cell diameters of control and TGFβ1-treated cells, respectively. These data were obtained from Coulter Counter measurements and interpolated by polynomials. Positions of maxima for the polynomials are indicated. Note the increase in the peak diameter of TGFβ1-treated cells.

motility in these cultures was again gradual and appeared to be no more rapid than with unconditioned TGFβ1-containing medium. Again the best-fit polynomial curve of third order (black line) gave no evidence to suggest that the increase is not a smooth process (one of the 95% confidence intervals is missed by the curve but we would expect one in twenty to be missed by random chance). Moreover, the time-averaged speed of 29.1 μm hour^{-1} was not significantly different from that with unconditioned TGFβ1-containing medium (ANOVA: P>0.1). These observations indicate that the delayed response is not due to the slow accumulation of a secondary released factor.

The TGFβ1-induced increase in motility is cell-cycle dependent

An alternative explanation of the gradual onset of the motile response to TGFβ1 is that the increase in motility might be cell-cycle dependent i.e. only cells that have originated from a division in the presence of TGFβ1 become more motile. The time taken for the response to reach a plateau (Fig. 3A) suggests that this might be the case since 15 hours is sufficient for most cells to have entered mitosis. Fig. 3C shows, for the same cells whose speed analysis was given in Fig. 3A, the percentage of the recorded cells that are known to have originated from a division during the recording period. The time course for the untreated cells (black points) is similar to that for the TGFβ1-treated cells (green points) and reaches a maximum before 15 hours in both cases. These curves never reach 100% (and even show a decline) because motile cells enter and leave the recording field and so we do not have cell-cycle information on all the cells at any one time. The curves also appear to show that there were no divisions during the first four hours which may indicate a slow recovery from plating. It arises in part, however, because this is not a record of divisions but of the appearance of isolated cells after division. Following mitosis, the two cells remain in contact with each other for up to an hour or more while they spread and flatten onto the substratum and it is only after they have started to migrate that they separate and can be recorded as isolated cells. Nevertheless, the time course of the increasing number of cells arising from division in the presence of TGFβ1 (Fig. 3C, green points) matches that of the gradual increase in mean speed of these treated cells (Fig. 3A, green points). We conclude that, if cells arising from division during TGFβ1 treatment are more motile, then the increasing number of these cells could explain the gradual increase in mean motility.

We next determined whether cells that have arisen from division during TGFβ1 treatment are indeed more motile than those that have not. For this, we analysed the motility of a subset of the untreated and treated cells whose stage in the cell cycle was known. These were cells that had been monitored continuously from the start of recording to the first division (mother cells) and their offspring during the next interphase (daughter cells). Cells that never entered mitosis nor arose from mitosis during the time that they remained within the recording field were thus excluded from this analysis. There were 87 mother cells and 162 daughter cells.

Fig. 3D shows the motility of the mother cells during the course of recording. In comparison with the total population of

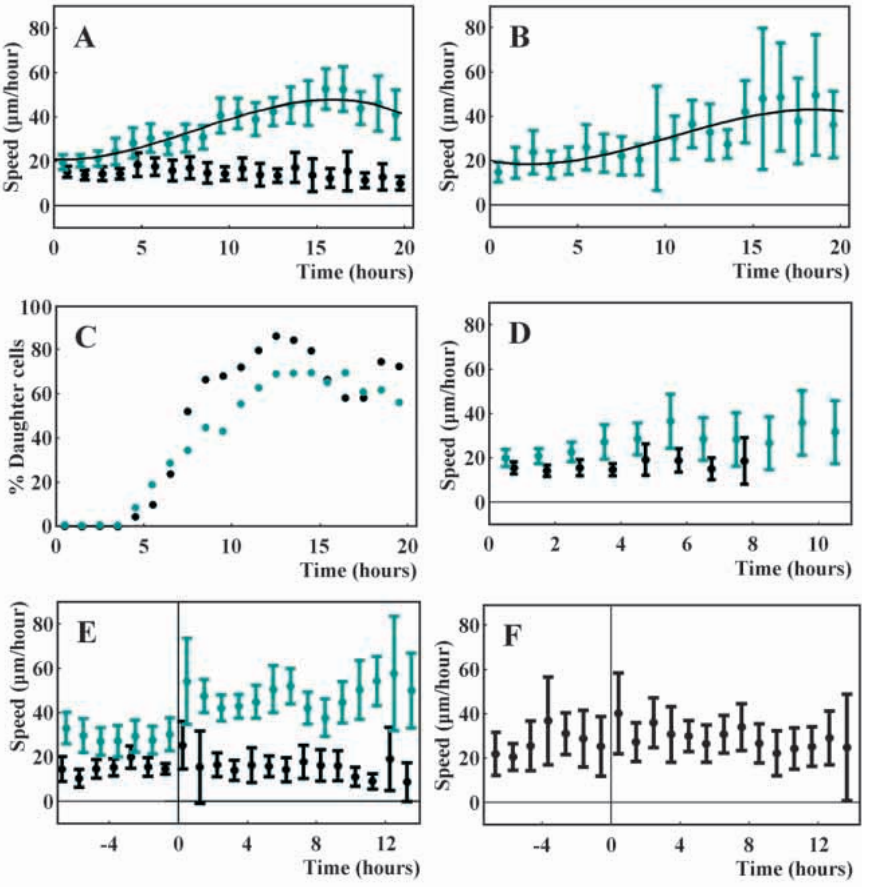

Fig. 3. (A) Time courses of speed based on analysis of four control recordings (black) and six recordings with additional TGFβ1 (green). (B) Time course of speed achieved by CCl64 cells that have been treated with conditioned medium. Time courses of treated cells in A and B are interpolated by polynomial curves demonstrating similar smooth processes. (C) Relates to A and represents proportion of cells in the observation field that are known to have originated from recorded divisions at each hour during the recordings with control (black) and TGFβ1-treated (green) cells. (D) Time course of speed in mother cells in the absence (black) or presence (green) of additional TGFβ1. (E) Speed of mother and daughter cells plotted against time after cell division (negative values specify time before cell division). Note an almost instantaneous increase in speed of daughter cells after mitosis. Also note the difference between maximum speed attained by mother cells and daughter cells. (F) Speed of CCl64 cells pulse-treated with TGFβ1 as a function of time after cell division. Error bars represent 95% confidence intervals.

cells (Fig. 3A), it is clear that the mother cells alone do not show such a dramatic response to TGFβ1. Nevertheless, there was a significant response and the time-averaged speed was 26.1 μm hour^{-1} in the TGFβ1-treated mother cells compared to 14.9 μm hour^{-1} in the control mother cells (ANOVA: $P<0.01$). Fig. 3E presents the motility of both the mother and daughter cells as a function of time relative to cell division. In the case of the untreated cells (black points), there was clearly no significant difference in mean cell speed before and after division (mothers: 14.9 μm hour^{-1}; daughters: 14.1 μm hour^{-1}; ANOVA: $P>0.1$). In contrast, the TGFβ1-treated cells showed a dramatic and highly significant difference in mean cell speed between mothers and daughters (mothers: 26.1 μm hour^{-1}; daughters: 45.1 μm hour^{-1}; ANOVA: $P<0.01$). It appears in the figure that the daughter cells have a higher speed immediately following cell division but we cannot be sure of this because of limited data (large confidence intervals) within the first hour or two after division. This again arises because, following mitosis, the two daughter cells are still rounded up and in contact with each other; it is only after they have started to migrate that they separate and can be recorded as isolated cells. We conclude that the dependence of the motile response on the cell cycle largely explains the gradual onset of the response.

In a new series of experiments, we finally investigated whether the higher speed of daughter cells depends on the continued presence of TGFβ1. Four cultures were exposed to TGFβ1 for 2 or 4 hours before recording and motility was then recorded for 20 hours in medium without added TGFβ1. Thus none of the daughter cells in these cultures had been exposed to TGFβ1, although the mother cells from which they had arisen had all been exposed at some stage. In order to exclude any cells that might have arisen from division in the presence of TGFβ1 before recording began, we eliminated from the analysis any cells (and their offspring) that did not divide during the first 10 hours of recording. Fig. 3F shows the motility of both the mother and daughter cells as a function of time relative to cell division. In this case, there was no clear difference in mean speed between mothers and daughters (mothers: 26.9 μm hour^{-1}; daughters: 25.2 μm hour^{-1}; ANOVA: $P>0.1$). Neither mothers nor daughters had a significantly different mean speed from the mothers that had been treated continuously with TGFβ1 in the previous experiments (ANOVAs, mothers: $P>0.1$, daughters: $P>0.1$) but both had significantly higher speeds than the untreated cells (ANOVAs, mothers: $P<0.001$, daughters: $P<0.001$). Thus it appears that administering a pulse of TGFβ1 during interphase does result in an elevated level of motility that is sustained for many hours and inherited by the offspring of the treated cells. However, we cannot rule out the possibility that this elevated level is due to residual TGFβ1 left in the system after gently washing the culture. Yet we can conclude that daughter cells do not have a significantly higher speed than their mothers if TGFβ1 is removed from the medium before division of the mothers.

TGFβ1 affects cell spreading and dynamic morphology

We have previously found that the spreading of different cell types is highly dependent on cell mass and that spread area generally shows a sharp upper limit which is directly proportional to mass (Dunn and Zicha, 1995). These CCl64 epithelial cells are no exception and Fig. 4 shows trajectories of the spread area versus mass for the whole populations of untreated (Fig. 4A) and TGFβ1-treated cells (Fig. 4B). The treated cells occasionally reach, but never exceed, an area-to-mass ratio of 7.78 μm^2 pg^{-1} as shown by the sloping lines in both figures. In contrast, the untreated cells cannot spread themselves so thinly and rarely approach close to this limit. Fig. 5A shows that the time averaged value of area-to-mass ratio is also much higher in the case of the treated cells (control cells: 3.2 μm^2 pg^{-1}; TGFβ1-treated cells: 4.4 μm^2 pg^{-1}; ANOVA: $P<0.01$).

The question arises whether cell speed depends on cell spreading in untreated cells so that the higher speed of treated cells is simply a consequence of their higher spreading. In fact we found in a linear regression model of speed versus spreading in the untreated cells that the slope of the regression line is only 1.26 μm hour^{-1} increase in speed for each 1 μm^2 pg^{-1} increase in spreading. This would only account for a 1.51 μm hour^{-1} increase in speed in treated cells which is not nearly enough to account for the increase of 21.7 μm hour^{-1} that we observed.

Another effect of TGFβ1 treatment is that the cell nucleus tended to become more peripherally situated. This asymmetry was quantified in terms of the cellular mass distribution as the mean distance between the mass centroid and the area centroid (Fig. 5B) and was highly significantly increased by TGFβ1 treatment (control cells: 1.5 μm; TGFβ1-treated cells: 2.8 μm; ANOVA: $P<0.01$). In contrast, the two parameters for describing cell shape, elongation and dispersion, were not significantly affected by TGFβ1-treatment. Mean elongation (Fig. 5C) of control cells was 1.13 and of TGFβ1-treated cells 1.15 (ANOVA:

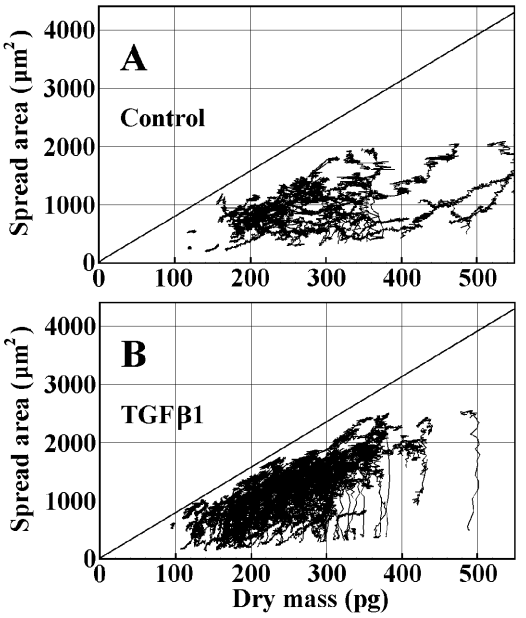

Fig. 4. Pooled trajectories of control (A) and TGFβ1-treated (B) cells from DRIMAPS recordings plotted as spread area versus dry mass. These plots demonstrate a sharp upper limit on the spread area which is directly proportional to mass. TGFβ1 increases this upper limit as can be seen by comparison with the diagonal reference lines.

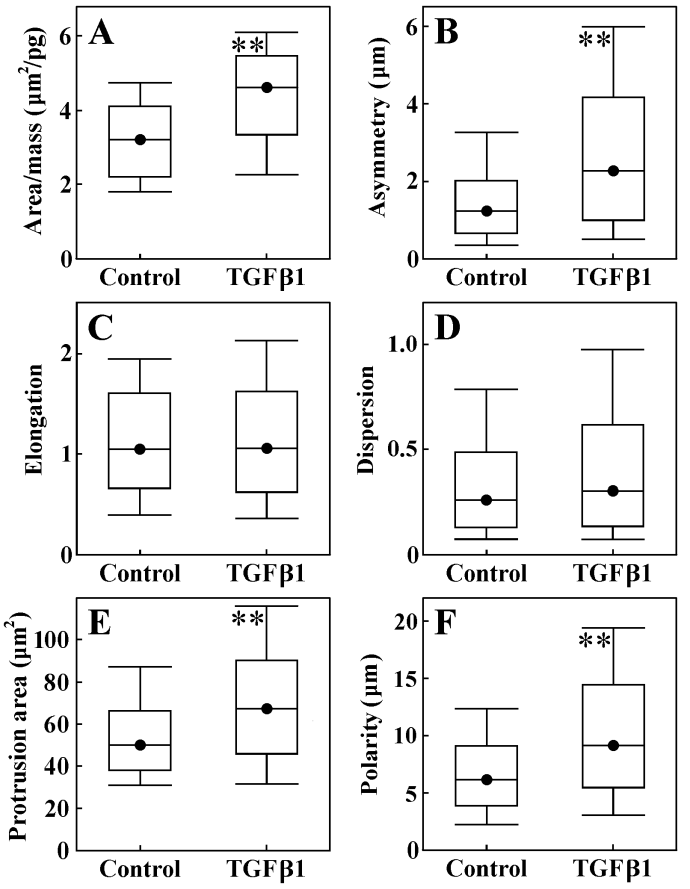

Fig. 5. 'Box and whisker' plots of cell spreading and dynamic morphology for control and TGFβ1-treated cells. Black dots represent median values, the boxes span 50% of the data and whiskers span 90% of the data. The two asterisks indicate a significant difference from the control of $P<0.01$ in an ANOVA test. (A and B) Significantly increased spreading (area-to-mass ratio) and asymmetry of TGFβ1-treated cells. (C and D) No significant effect (ANOVA $P>0.1$) of TGFβ1 on cell shape described by elongation and dispersion. (E and F) TGFβ1-induced increases of protrusion and polarity, respectively.

$P>0.1$). Mean dispersion (Fig. 5D) of control cells was 0.34 and of TGFβ1-treated cells 0.44 (ANOVA: $P>0.1$).

Besides the speed measurements, the motility of the cells was also determined by measuring their areas of protrusion and retraction and their polarity. Compared with control cells, the TGFβ1-treated cells had a significantly increased mean area of protrusion, from 50.3 μm^2 to 67.9 μm^2 (ANOVA: $P<0.01$) and of retraction, from 50.0 μm^2 to 67.2 μm^2 (ANOVA: $P<0.01$). The analysis of protrusion is shown in Fig. 5E but retraction is not shown since it is almost identical. Proportionally, cell polarity showed an even greater increase on TGFβ1 treatment from 6.8 μm to 10.4 μm (ANOVA: $P<0.01$) as shown in Fig. 5F.

We have previously found a theoretical relationship which states that the speed of a cell is equal to relative protrusion times polarity where relative protrusion is the area of protrusion divided by the area of the whole cell (Dunn et al., 1997b). This relationship is exact when speed is measured as the displacement of the cell's area centroid and when protrusion area equals retraction area. Even so, it should be a reasonable approximation for the displacement of the mass centroid under conditions when the cell's total area is not changing very rapidly. Here we found that the effect of TGFβ1 treatment was almost to double the product of relative protrusion and polarity from 0.37 μm to 0.71 μm (ANOVA: $P<0.001$). In a linear regression model of speed versus relative protrusion times polarity in the untreated cells, we found that the slope of the regression line is 49.2 μm hour^{-1} increase in speed for each unit increase in relative protrusion times polarity. Therefore the observed increase in relative protrusion times polarity in treated cells could account for a 16.7 μm hour^{-1} increase in speed solely on the basis of this relationship. This accounts for a large part of the observed increase in speed of 21.7 μm hour^{-1}.

DISCUSSION

TGFβ1 does not alter the rate of increase in cell mass and therefore causes a progressive increase in cell size

The rate of increase in mass of the mink lung cells was similar in control and TGFβ1-treated cells at about 0.07% minute^{-1}. Thus the increased cell cycle time of TGFβ1-treated cells that we have found in a previous study (Kramer et al., 1994) is not explained simply by a reduction in their rate of mass increase. This finding is consistent with a report by Like and Massague (1986) where it was shown that phosphorylation of S6 kinase, a phosphorylation coupled to the stimulation of protein synthesis by growth factors like EGF and PDGF, was unaffected by TGFβ1-treatment. These data clearly show that not only does TGFβ1 not interfere with the early growth factor signalling events (Chambard and Pouyssegur 1988; Kramer et al. 1991; Like and Massague, 1986) it also leaves certain growth factor-induced events entirely uninterrupted. These data clearly show that an overall diminution of the growth factor signal is not the way TGFβ1 increases the cell intermitotic time.

A consequence of the increased cycle time coupled with an undiminished rate of increase in mass is that the treated cells must become progressively larger. We confirmed this by Coulter-counter measures of cell diameter which gave values of 14.5 μm for untreated cells compared with 17.0 μm after a 38-hour treatment with TGFβ1. From this we calculated that the treated cells had a 61.1% larger mean cell volume and a correspondingly larger mean cell mass than the controls assuming unchanged mass density. This is approximately what we would expect from an extended intermitotic time of 6 hours (Kramer et al., 1994). Cells growing in mass exponentially at a rate of 0.07% minute^{-1}, and spending six hours longer in each cycle than control cells growing at the same rate, would be 28.7% heavier after one division and 65.5% heavier after two divisions. It is probable that a large majority of the cells would have undergone exactly two divisions during the 38-hour treatment. Thus the observed size increase is consistent with a 6-hour increase in cycle time and confirms that the growth rate is undiminished by TGFβ1 treatment.

TGFβ1 induces a cell-cycle-dependent increase in motility

The gradual development of increased mean speed in the population of TGFβ1-treated cells is largely explained by our

finding that cells arising from a division in the presence of TGFβ1 move much faster than cells that have not yet divided in the presence of TGFβ1. It is unlikely that the response is additionally delayed by a need for the cells to release autocrine factors since the time course of the response was closely similar in TGFβ1-containing medium that had been pre-conditioned by cells. In fact there is little evidence in our data that the response of individual cells is gradual: the treated mother cells have a higher speed than untreated cells from the outset of the experiment and the much higher speed of daughter cells is fairly constant with time after division. We conclude that the apparent gradual onset of the response is mainly a population effect brought about by a two-stage increase in cell speed: an initial response on the addition of TGFβ1 and a further response after cell division has occurred.

Administering a pulse of TGFβ1 for 2 or 4 hours during interphase is sufficient to induce the first stage of the two-stage response which results in a doubling of cell speed that is sustained until the cell divides. This pulse treatment appeared to be as effective at inducing the response as a continuous treatment throughout interphase. Surprisingly, the pulse treatment is 'remembered' by the cells' daughters after division which maintain the elevated speed for several hours. We think it unlikely that this memory mechanism is due to residual TGFβ1 adsorbed to the substratum, and picked up by the cells as they crawl over it, since the pulse-treated cells do not show the second stage of the response. It appears more likely that some biochemical changes occurring during the signal cascade within the cells can persist for several hours in the absence of new signals and be inherited by the daughter cells after division.

The second stage of the response results in a further increase in mean speed to about three times the basal level. This occurs only after cell division and requires that TGFβ1 must be present in the medium at or just before division. This apparently sudden increase in speed following cell division is not a normal phenomenon since the untreated cells show no sign of this. Furthermore, our pulse-treatment experiments clearly show that there is no significant increase in speed on division when TGFβ1 is no longer present in the medium. We therefore postulate the presence of a G_2 cell cycle check point; a checkpoint that determines cell motility in the next generation of cells.

Mechanism of the motile response to TGFβ1

Our results show that the increase in speed following treatment with TGFβ1 has two components, an increase in the areas of protrusion and retraction and an increase in polarity. The increase in polarity is particularly striking and may account for the increase in asymmetry that we observed since a more polarised cell has more clearly defined front and rear ends and the bulky nuclear region of a cell tends to become displaced towards the rear. The control and mechanism of cell polarity is still poorly understood although microtubules are thought to be involved (Bershadsky and Vasiliev, 1993). The control and mechanism of cell protrusion, on the other hand, is becoming better understood and it appears that the formation of lamellipodia (which are the main type of protrusions observed in the cells that we studied) requires the activation of Rac (Nobes and Hall, 1995). Our observation of increased protrusion of the cell margin may therefore implicate the Rho family of GTPases as downstream effectors of TGFβ1 receptors.

TGFβ1 induces an increase in cell spreading

Our observation that cell spreading is increased by TGFβ1 treatment implies a change in cell/substratum or cell/matrix interactions. In fact TGFβ1 is known to increase the levels of secretion of matrix components such as fibronectin, thrombospondin and the plasminogen activator inhibitor-1 (Laiho et al., 1991) and it was also shown that it affects integrin expression in human osteosarcoma cells (Heino and Massague, 1989). The question arises of whether the enhanced speed is a direct result of this increased spreading. This seems unlikely since increased adhesion to the substratum generally results in a reduced speed, although this is not always the case (Palecek et al., 1997). Furthermore, we have demonstrated using a regression model that the higher speed of treated cells is not a simple consequence of their higher spreading. Nevertheless, it is still possible that the two responses might have a common cause. A general increase in matrix deposition and a change in integrin expression induced by TGFβ1 treatment could enhance the mean spreading of cells and also affect their mean speed; our failure to observe a strong statistical relationship could then be explained since cells that have an enhanced spreading need not be the same individuals as those that have an enhanced speed. Even so, the generally increased spreading that would result from such a mechanism cannot explain the sudden increase in speed observed to follow cell division. Whatever the relationship, if any, between the speed and spreading responses, it is clear that one response is not simply a direct effect of the other as it is in the case of the relationship of speed to protrusion and polarity.

The relation of the motile response to cell cycle progression

In the previous study in which we analysed TGFβ1-induced cell-cycle changes (Kramer et al., 1994), the response was entirely post-mitotic which suggests a relationship to the post-mitotic increase in cell speed. The time delay to the onset of the first mitosis in the presence of TGFβ1 was not changed compared to control cells and inhibition of the cell cycle only became apparent after the cells had passed through mitosis in the presence of TGFβ1 (Kramer et al., 1994). We took these data to mean that TGFβ1 did not effectively delay the ongoing cell cycle. More recent unpublished observations (Kramer) in which cells were recorded for several hours prior to the addition of TGFβ1 revealed that 90% of the cells in early G_1 at the time of addition of TGFβ1 did not respond with a significant increase in cell cycle time, whereas their daughter cells did. Thus mink lung epithelial cells cannot fully respond to the TGFβ1 signal immediately: high motility and cell cycle inhibition only become manifest after cells have past mitosis.

Again, a possible link between the two responses is cell/matrix interaction. Evidence is accumulating that cell-cycle progression is dependent on cell/matrix interactions: changes in integrin expression have been shown to change cell-cycle progression (Meredith et al., 1995) and contact with the extracellular matrix has also been shown to be vital for cell-cycle progression (reviewed by Assoian, 1997 and Giancotti, 1997). However, despite the evidence that TGFβ1 treatment does enhance matrix secretion (Laiho et al., 1991) and changes integrin expression (Heino and Massague, 1989), it is unclear whether this would result in an increased cycle time and, for the reasons given in the last section, it seems unlikely that it would result in the cell-cycle-dependent speed changes that we observed.

Homology with dorsal closure in *Drosophila*

The five features induced by TGFβ1 in mink lung epithelial cells, namely increased intermitotic time, cell asymmetry, flattening, increased protrusion formation and increased cell size, resemble cellular events that take place during dorsal closure in *Drosophila*. The dorsal surface of the *Drosophila* embryo is formed by the migration of the lateral epithelial cells to cover the amnioserosa. Expression of decapentaplegic (DPP), a TGF-β homologue, in the leading edge of the dorsal epithelium, is identified as necessary for this process. During dorsal closure the most dorsal cells release DPP which then induces morphological changes in the lateral ectodermal cells. These cells flatten, increase in size and stretch in the direction of the dorsal ridge thus closing the epithelial layer (Glise and Noselli, 1997; Hou et al., 1997; Riesgo-Escovar and Hafen, 1997a,b). The changes occurring during dorsal closure and the observed TGFβ1-mediated changes in mink lung epithelial cells could be a consequence of a conserved pathway that directs embryological development but in a later developmental stage may have a role in, for instance, wound healing. Because of homology with dorsal closure, *Drosophila* studies could help to provide further possible genetic clues in dissecting the TGFβ1 signal transduction pathways in mammalian cells.

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