Clinical & Experimental Metastasis **18:** 589–597, 2001.

Migratory activity of human glioma cell lines *in vitro* assessed by continuous single cell observation

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Received 15 August 2000; accepted in revised form 7 June 2001

Key words: brain tumor, cell culture, glioma, invasion, migration, videomicroscopy

Abstract

A new migration assay, the time-lapse individual cell migration assay (TIM-assay), was developed, which allows the observation of cells over 24 h under controlled conditions. Using this technique, the migratory behavior of 8 human glioblastoma cell lines *in vitro* was studied. Special features are simultaneous documentation of migratory parameters of individual cells, i.e., migration velocities and migration paths of individual cells. Migration velocity for cell populations of the same cell line ranged from 0 to 24 μm/h. The migration paths were examined for being directional. Two thirds of all cells showed directional migration. Migration paths were further classified according to visual judgements for being linear, oscillating or mixed. The migration index had a mean of 91%. The presented TIM-assay allows the assessment of several new parameters, that may be useful to identify subgroups of gliomas with different biological characteristics.

Introduction

Gliomas are the most common primary cerebral neoplasms in man. Features that count for the poor outcome of patients harboring such tumors include highly invasive growth characteristics, the lack of effective barriers in the brain, and limited response rates to radiation and chemotherapy. The malignant behavior of gliomas includes proliferation and invasion, the latter being a well-controlled coordination of adhesion to neighboring cells and extracellular matrix proteins (ECM), degradation of ECM, and migration. Glioma cell migration has been shown to be an active process following specific anatomic structures such as the perivascular space and white matter tracts, such as the optic radiation and corpus callosum [1].

Furthermore, an inverse correlation between proliferation and migration has been postulated in such a way that cells on migration permissive substrates show reduced proliferative activity and *vice versa* [2].

Different methods have been introduced for the evaluation of glioma migration *in vitro*. The 'monolayer-migration assay' [3, 4] and the 'radial dish assay' [5] measure migration as a net change in the area covered by cells over time, when seeded on a defined area. Further aspects of migration can be analyzed with the transwell assay [6], which quantifies cell migration through a membrane of a defined pore size.

There are only very few methods capable of examining single cell behavior *in vitro*. These are the 'videomicroscopy' by Chicoine and Silbergeld [7], a method by Hegedüs et al. [8], and one by De Hauwer et al. [9]. In this article we present a newly developed migration assay, named 'time lapse individual cell migration assay' (TIM-assay), that enables continuous observation and analysis of the migration path of human glioma cells *in vitro* in a controlled environment [10]. We developed new methods, that give easy-to-use parameters characterizing the migration path (MP). Initially, the MP of individual cells is tested for being directional or random. Subsequently, the MP is characterized using the migration coefficient (MC). Differences in the migration path may be of biomedical relevance in terms of invasiveness of certain tumors. Especially a high degree of directional movement in glioma cells may count for the fast development of tumor recurrence in the surrounding brain tissues and the poor outcome of patients with these tumors.

Materials and methods

Cell culture and growth curves

Three cell lines Mz18, Mz21 and Mz35 were established from brain tumor specimens obtained during craniotomies for tumor resection. Specimens were split with one

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part sent for histopathological evaluation and one part for culture. Histopathological characterization included hematoxylin/eosin staining and immunohistochemical expression of glial fibrillary acidic protein (GFAP). Each of these cell lines derived from tumors classified as glioblastoma multiforme (GBM) WHO IV (Table 1).

Surgical specimens were transferred immediately from the operation field into sterile Dulbecco's modified Eagle's medium (DMEM), minced to a slurry with scalpels, suspended in 0.9% NaCl and centrifuged at 630 *g* three times. Cells were then resuspended in culture media with addition of 1 g/l D-glucose, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamin and 3.7 g/l $NaHCO_3$ and plated in 90 mm petri dishes. Cells were kept in DMEM with 20% fetal calf serum (FCS) and incubated at 37 °C in a humidified 10% CO_2 atmosphere.

Five well-characterized human glioma cell lines (TP378 MG, TP483MG, G-44, G-168, G-270) [11, 12] were propagated in monolayer cultures in minimal essential medium (MEM) with 10% FCS. Cells were passaged at regular intervals depending on their growth characteristics using 0.25% trypsin (Seromed Biochrom KG, Berlin, Germany). At certain passages cells were stored in a dimethylsulfoxid solution (DMSO) in liquid nitrogen for later use.

Growth curves were established with an initial concentration of 30 cells/μl. Counts of three wells were performed for each cell culture using a Neubauer's counting chamber at 24 h intervals.

Time lapse individual cell migration assay (TIM-assay)

For the TIM-assay, 3×10^4 cells/ml were plated in 90 mm tissue culture treated petri dishes (Greiner, Germany). After 24 h, dishes were placed under an inverse phase-contrast-microscope (Nikon Diaphot-TMD, Nikon, Düsseldorf, Germany) equipped with a styropor isolated lucite cube enclosing the stage. The petri dishes were maintained in the controlled-atmosphere lucite box and randomly selected fields of cells were observed for a time period of 24 h. At 10× magnification, microscopic fields of about 500 μm × 500 μm were recorded with a CCD camera (Sony, Japan). Images were captured and digitized on a personal computer every 600 sec in order to generate a time lapse video. The above time-interval was chosen empirically as a compromise between resolution in time and consumption of hard disk space. For quantification of the migratory activity, consecutive positions of nuclei in all cells were marked by mouse click (referred to as 'tracking') using an image analysis program (NIH-Image: National Institute of Health, USA). For each cell line about 20 to 25 cells were examined per experiment.

Homeostasis inside the incubation cube at a temperature of 37 °C, a pH of 7,4 and an osmolality of 330 mOsm/kg H_2O was provided by a heating coil, a mini-fan, a CO_2 insuflation system, and humidification of the atmosphere to prevent culture media evaporation. Temperature was controlled by a thermostat and continuously monitored by two independent temperature sensors (Heraeus, Hanau, Germany; Shiley, Irvine, USA). CO_2 concentration was controlled and continuously monitored by a CO_2 regulation unit (Heraeus, Hanau, Germany). In the early phase of the experiments, additional testing using CO_2 test tubes (Dräger, Lübeck, Germany) was performed. The pH and osmolality of the culture medium was documented before and after each TIM-assay using the ABL blood gas analysis system (Radiometer, Willich, Germany) and an osmonometer (Osmomat 030, Gonotec, Berlin, Germany). In selected TIM-assays, temperature, pH, pCO_2 and pO_2 were continuously measured for 24 h using the Paratrend 7 system (Diametrics, Buckinghamshire, UK).

Motility parameters

Cells were recognized as 'migrating' when the migration velocity (MV) was greater than 30 μm/12 h. This threshold results from the observation that some big cells just move around their cytoplasm but do not actually migrate. As the average diameter of a big cell was 30 μm, this was taken as the lower threshold of migration. Cells that could be tracked for intervals less than 8 h due to leaving the microscopic field were excluded from further evaluation, since results for those cells were found to differ significantly in MV from cells observable for more than 8 h (data not shown).

For Mz18, Mz21, Mz35 and TP378MG the TIM-assay was performed in triplicate; for all other cell lines a single TIM-assay was used for estimation of migration and proliferation parameters.

Migration velocity (MV)

The migration velocity (MV) was calculated from the distance of two consecutive cell coordinates over time (600 sec) and referred to as 'actual velocity' (v_{act}). The median of all v_{act} of one cell was defined as v_{cell}. The median of all v_{act} derived from all migrating cells of a single assay was defined as v_{tumor} (Table 1). Median velocities were displayed as distances per hour. The median as parameter for the 'central tendency' of the raw data was used because of its relative robustness against extreme values which could be seen quite early in the investigation. In further statistical calculation, the mean and standard deviation (SD) of the so-defined velocities v_{cell} and v_{tumor} were calculated.

To analyze whether a systematic change of MV occurs over time, linear regression analysis was performed for the velocity slope of each cell. A constant MV was assumed if the 95% confidence interval of the linear regression included zero. Reproducibility of the parameter 'migration velocity' (MV) was tested using variance-analysis. v_{cell} of up to four independently performed TIM-assays of the cell lines TP378MG and Mz21 were tested for being not different by using high alpha-error. Results were called reproducible when alpha was more than 0.20.

Migration path (MP) and migration coefficient (MC)

The MP was visualized by plotting the consecutive x/y-coordinates of each cell of a TIM-assay (Figure 1). The length of the MP was calculated by adding up consecutive distances of each cell of a TIM-assay.

Table 1. Data of newly established cell lines.

ID	Age	Sex	Position	Tumor volume	Histology	WHO grade	GFAP	Passage
Mz18	72	Male	Right occipital	30 ccm	GBM	IV	+++	82
Mz21	72	Female	Left parietal	18 ccm	GBM	IV	++	100
Mz35	39	Male	Left frontal	30 ccm	GBM	IV	+	72

Intensity of staining for GFAP was classified as slightly positive (+), moderately positive (++) or strongly positive (+++).

Table 2. Newly introduced motility parameters assessed by TIM-assay.

Migration velocity (MV)	
v_{act}	Migrated distance per 10 min observation time [μm/h]
v_{cell}	Median of v_{act} of one cell
v_{tumor}	Median of v_{act} of one cell line
Migration path (MP)	Plotting of consecutive x/y-coordinates
Migration coefficient (MC)	$MC = \frac{\text{radial distance migrated}}{\text{length of MP}}$
Migration index (MI)	$MI = \frac{\text{cells migrating}}{\text{start number of cells}} \times 100\%$

v_{act} = actual velocity, v_{cell} = single cell velocity, v_{tumor} = velocity of tumor cell population.

To distinguish whether the cell motion was directional or not, vectors achieved by connecting sequential coordinates were transformed into unit vectors. From these, the angles of each vector with the positive x-axis in the anti clockwise direction were determined. Angles for each cell were tested for being non-uniformly distributed by applying the Rayleigh test [13]. Cells classified as having non-uniformly distributed angles (i.e., H_0 rejected) were referred to as 'directional' migrating cells. Cells classified as having uniformly distributed angles (i.e., H_0 not rejected) were referred to as 'randomly' (i.e., non-directional) migrating cells.

The evaluation of MP of directionally migrating cells of all TIM-assays resulted in the subjective recognition of at least three different patterns of migration paths (Figure 1). Two raters independently classified the MPs of all cells as oscillating, linear or mixed according to their appearance. Interobserver agreement was tested with the kappa test. The kappa value measures the proportion of the agreement beyond that to be expected by chance.

The maximum radial distance migrated was calculated, which gives the net movement from the point of origin for each cell. The migration coefficient (MC) was determined for directionally migrating cells as the maximum radial distance migrated (i.e., the maximum distance of each point of the MP related to the point of origin for each MP) divided by the distance migrated totally.

To analyze, whether the MC allows a differentiation between the visually determined patterns of MP's a hierarchical cluster analysis (between groups linkage and squared Euclidian distances) of the MC of all cell lines was performed. The results for a three-cluster solution were compared with results of the visual classification. Furthermore, the total cell displacement was calculated as the total distance migrated by each cell.

Migration index (MI)

The MI was introduced to define the proportion between migrating and non-migrating cells. The MI was calculated by dividing the number of migrating cells by the number of cells in the microscopic field at the beginning of each assay and multiplied by 100.

Results

Homeostasis of temperature, pH, osmolality

The temperature in the culture medium in petri dishes incubated in the cube was found to range from 36.8 to 37.2 °C. The pH ranged between 7.37 and 7.40 (SD = 0.05). Osmolality was found to differ from the desired value of 330 mOsm/kg H_2O not more than 10 mOsm/kg. A control 24-h online registration with the Paratrend 7 system confirmed these findings with a mean pH of 7.40 (SD = 0.02) and a mean temperature of 36.8 °C (SD = 0.07).

Observation time

No systematic changes in the v_{act} could be found in the majority of cells (61%) during 24-h assays. In the 42-h assays a decrease of v_{cell} after a mean duration of 17 and 18 h, respectively, was found for the cell lines TP378MG and Mz21. Despite the quite early reduction in v_{cell}, we chose an observation time of 24 h, for detailed tracking of the MP and counting of mitoses over 24 h.

Migration velocity (MV)

More than 250 cells were examined in the present study (Table 3). Cells that could be tracked for less than 8 h due to leaving the microscopic field were excluded from further evaluation, since results for those cells were found to differ

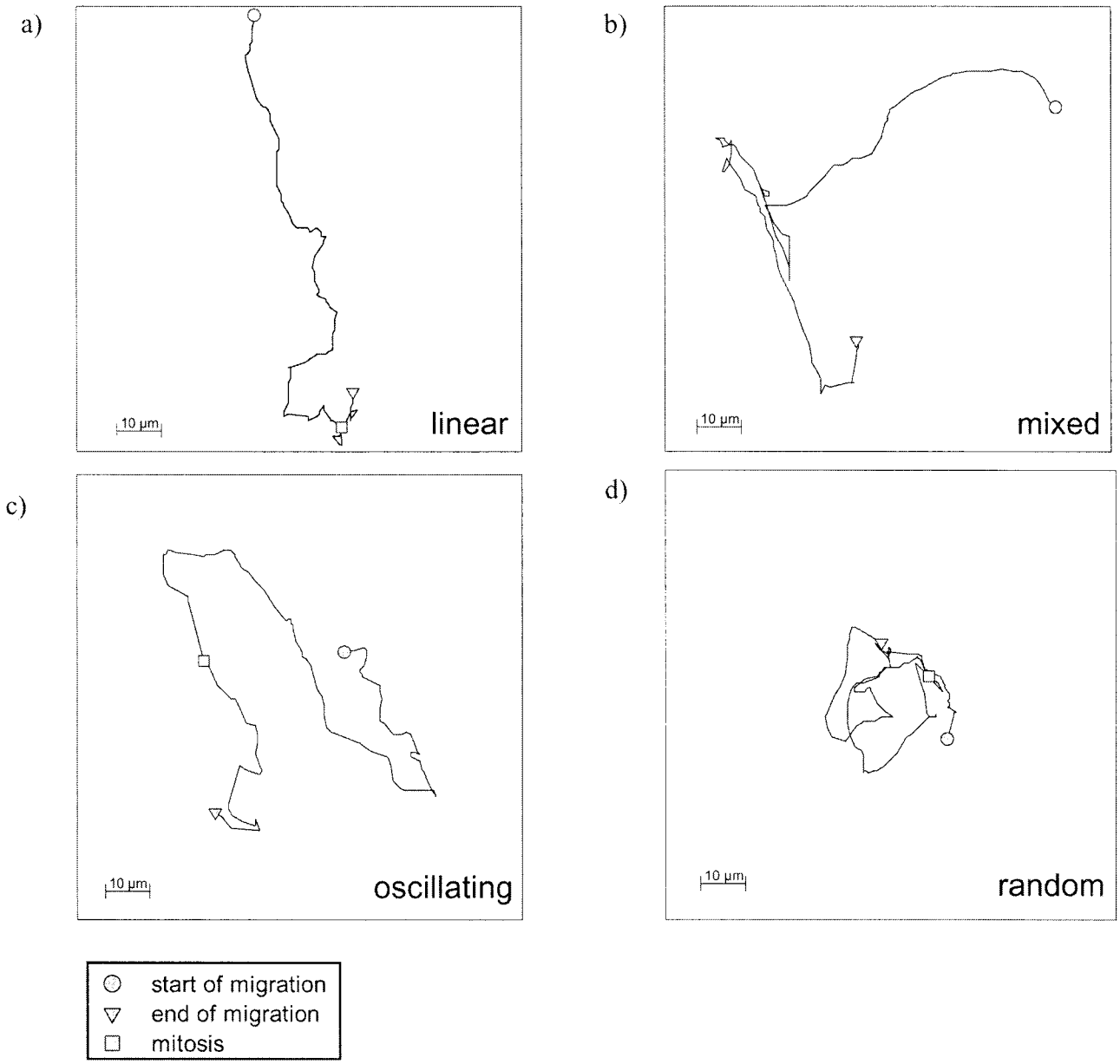

Figure 1. Migration path of 4 selected cells from cell line Mz35. First three graphs show examples for cells migrating directionally and having a linear (a), mixed (b) or oscillating (c) migration path according to visual classification; bottom right graph shows a cell migrating in a random way (d).

Table 3. Migration velocities and migration index (MI) of human glioma cell lines.

ID	Amount of cells	v_{tumor} [μm/h]	Range v_{act} [μm/10 min]	SD v_{tumor}	MI %
Mz18	36	18.5	0–56.1	6.7	78
Mz21	33	13.4	0–27.8	8.3	78
Mz35	55	12.7	0–22.4	4.9	99
TP378MG	58	8.5	0–23.4	5.6	90
G-44	21	0.0[a]	0–11.4	3.3	95
G-168	14	24.2	0–44.4	11.9	95
G-270	18	10.2	0–29.5	6.1	95
TP483MG	19	6.0	0–17.0	2.7	79

[a]Velocity of zero resulting from more v_{act} being 0 than > 0; v_{tumor} = median of all v_{act} derived from the same tumor, v_{act} = actual velocity, SD = standard deviation, MI = migration index.

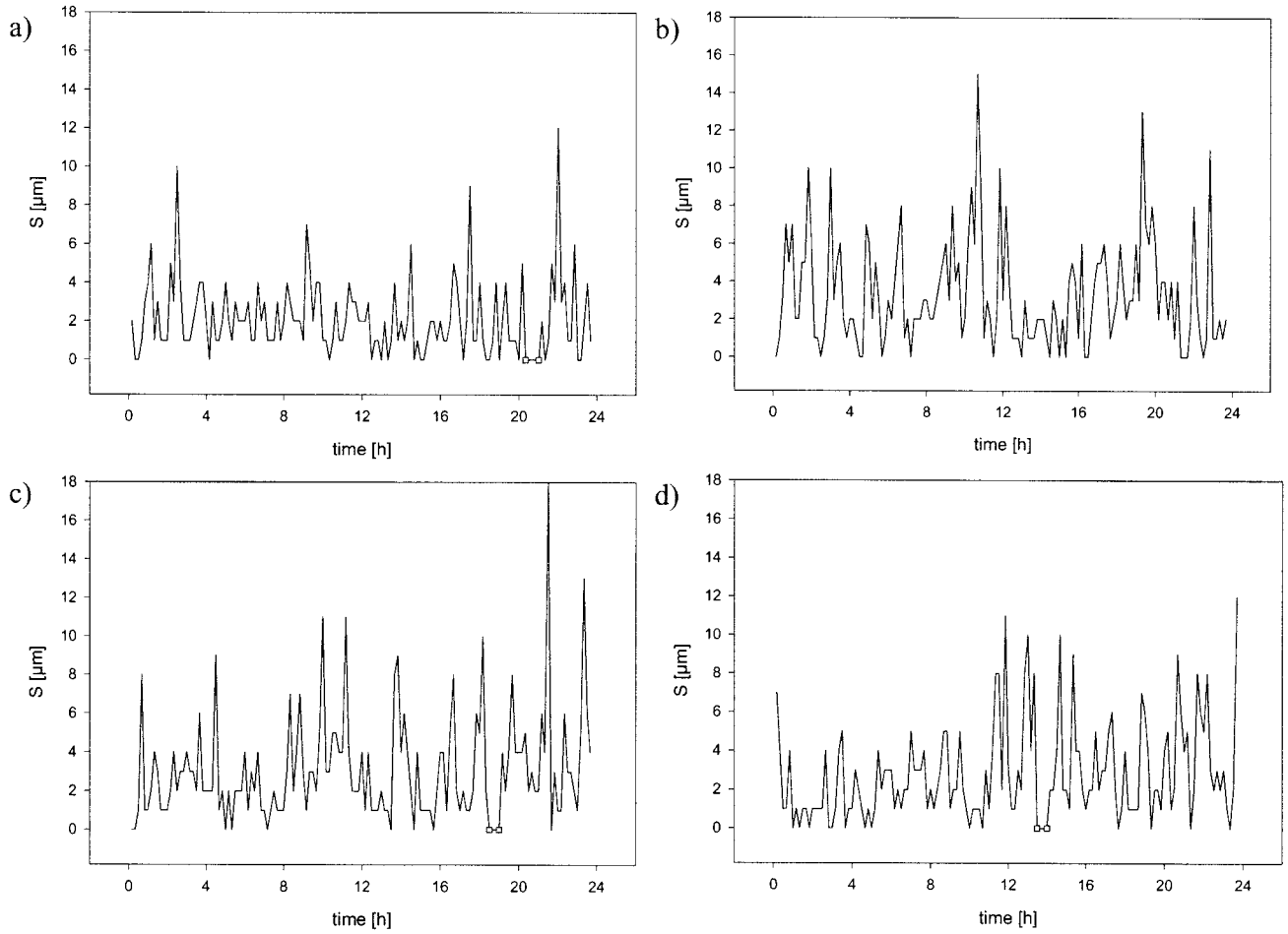

Figure 2. Plot of v_{act} over time. Graphs correspond to Figure 1. A. Actual velocity (v_{act}) of the linear cell. B. v_{act} of mixed type cell. C. v_{act} of oscillating cell. D. v_{act} of random migrating cell. Squares indicates mitosis.

significantly in v_{cell} from cells observable for more than 8 h (data not shown).

Evaluation of the MV revealed for the individual cells a v_{act} ranging from 0 to 56.1 μm/10 min, leading to a mean v_{cell} of 12.4 μm/h (SD = 7.96) ranging from 0 to 45 μm/h. (A velocity of 0 is resulting from the use of the median. If more than 50% of the values are 0, the median will be 0). For the individual cell lines, a mean v_{tumor} of 11.7 μm/h (SD = 7.45) ranging from 0 to 24.2 μm/h was calculated (Table 3).

The analysis of variances (ANOVA) of incremental cell velocities (v_{cell}) over the 24-h interval for Mz21 ($P = 0.42$) and TP378MG ($P = 0.23$) revealed no differences. This implies that the v_{cell} is a randomly fluctuating behavioral trait of the cell line. Despite the inconstant values of the v_{act} (0 up to 56 μm/10 min) (Figure 2), 61% of all evaluated cells showed no overall change in velocity; 23% showed a deceleration and 16% showed an acceleration over the observation time of 24 h (Figure 3). ANOVA of v_{tumor} of different glioma cell lines showed significant interindividual differences. G-168 had the highest v_{tumor} with 24.2 μm/h and G-44 the lowest v_{tumor} with 0.0 μm/h (Figure 4A).

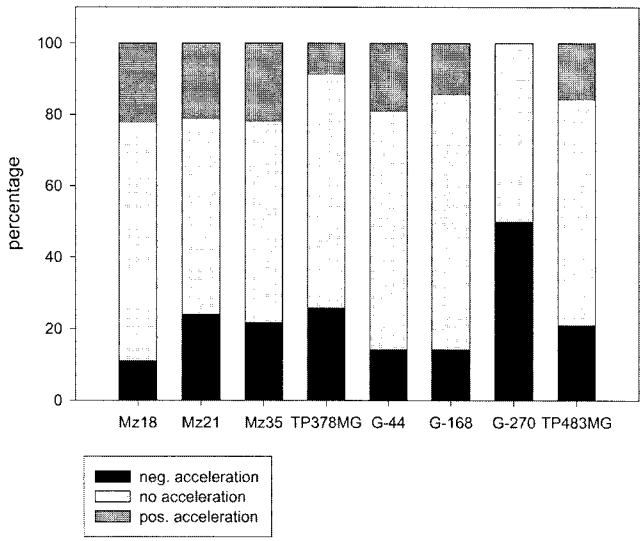

Figure 3. Acceleration of evaluated cells during TIM-Assay. Percentage of accelerating cells, demonstrating that at least 50% of cells show neither acceleration nor deceleration.

Migration path

The Rayleigh Test revealed that 67.7% of all cells, ranging from 50% to 89% for the individual cell lines, showed directional migration (Figure 5). The lowest percentage of directionally migrating cells was observed for the cell line Mz18 with 50%.

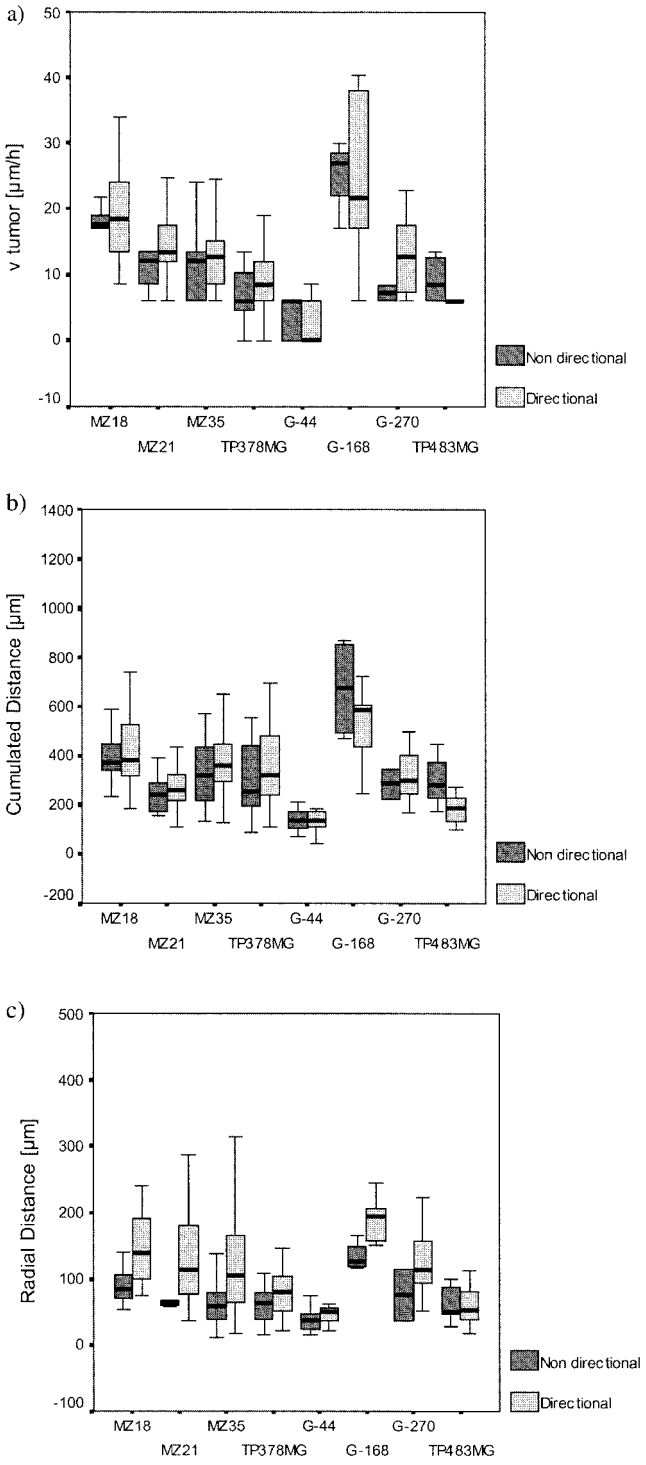

Figure 4. Tumor velocities (v_{tumor}), total cell-line displacement (cumulated differences) and radial distance for directionally and randomly migrating cells. Data are presented by boxplots. A. Distribution of tumor velocities (v_{tumor}), demonstrating highly significant interindividual differences. No significant differences were observed between directionally and randomly migrating cells. B. Distribution of cumulative distances for the different cell lines demonstrate highly significant interindividual differences as well as differences between directionally and randomly migrating cells. C. Distribution of radial distances for the different cell lines demonstrates highly significant interindividual differences as well as differences between directionally and randomly migrating cells.

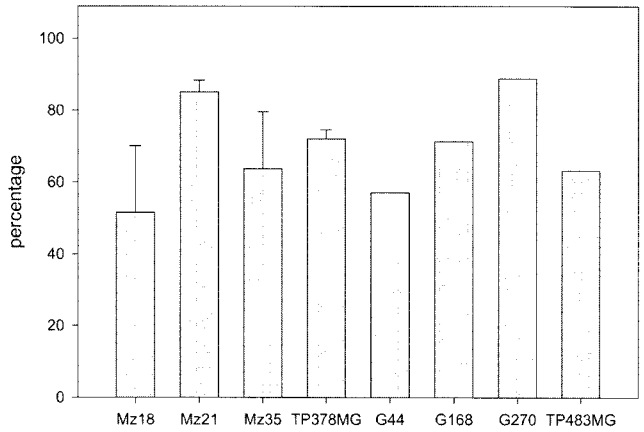

Figure 5. Results of Rayleigh-test for evaluated cultures. Percentage of cells classified as migrating directionally. At least 50% of evaluated cells showed directional movement.

No significant difference in v_{tumor} was found for directionally and randomly migrating cells ($P = 0.18$) (Figure 4A).

No significant difference in the cumulated distance (total distance migrated by each cell) for directionally and randomly migrating cells was found ($P = 0.63$) (Figure 4B). Comparing the maximal radial distance migrated by directionally migrating and randomly migrating cells, we found a significant difference ($P < 0.001$). Cells exhibiting a directional migration path showed in general a higher radial distance ($mean_{directional\ migration} = 114.2$, $mean_{random\ migration} = 72.4$) (Figure 4C).

The mean migration coefficient (MC), only calculated for cells classified as having a directional movement, was 0.35 (SD = 0.16).

Comparison of the classification of MPs performed by two raters revealed a good interobserver agreement ($\kappa = 0.85$) with 91% corresponding judgements. These results were selected for comparison with a three-cluster and two-cluster solution, respectively. There was a 75% correspondence for cells classified as linear by visual classification and classified in cluster 1 (low MC values) by a three-cluster solution. For cells classified as mixed and oscillating by visual classification, there was only a 15–45% congruence with three-cluster solutions for cluster 2 (medium MC values) and 3 (high MC values), respectively. The kappa test for all cell lines of the corresponding judgements and the cluster solutions therefore yielded a very low score (−0.09) just about chance level.

Migration index (MI)

The MI ranged from 78 to 99% (mean = 88.6; SD = 8.8). Mz 18 and Mz 21 were found to have the lowest MI with 78%, Mz35 the highest with 99% (Table 3).

Discussion

The TIM-assay was established in order to enable continuous single-cell observation of cell migration under controlled conditions over an extended period of time (up to

24 h). Using human glioma cell lines, migration was determined with this method by assessing migration velocity (MV), migration path (MP), and migration index (MI). A panel of eight cell lines was used to investigate the constancy of the assay parameters, i.e., temperature, pH and osmolality, as well as the reproducibility of the migration parameters, i.e., MV, MP and MI.

Using independent instruments and measurements, homeostasis of the culture media could be confirmed up to 24 h. Therefore, the developed incubator is capable of sufficiently supplying constant conditions for long-term observation of cell cultures. Presently, glioma cell migration is assessed with methods based on subsequent measurement of the enlarging area covered by cells over time, such as the 'radial dish assay' and 'monolayer-migration assay' [3, 5, 7, 14, 15]. Using this type of assay, resulting velocities are reported to range from 0.4 to 10.8 μm/h [16, 17].

Although the increase in area is thought to reflect pure cell migration as demonstrated by lack of proliferating cells in the peripheral zone by immunohistochemical methods, the influence of proliferation cannot be quantified in each assay [15]. Another major disadvantage of these methods is that measurements represent net changes in size and distribution of the migrating cell population but do not reflect movements of individual cells [18]. Therefore, only phenomena based on a global population of cells are registered. For example, the MV is thought to be underestimated with area-based tests, because it is very unlikely for cells to migrate in a straight centripetal manner. To prevent uncontrolled influence from cell proliferation, Chicoine and Silbergeld [7] used the anti-mitotic agent hydroxyurea with the 'radial dish assay' and reported no significant influence of hydroxyurea at a concentration of 10 mM on cell migration. However, careful testing using single-cell observation is necessary to confirm this hypothesis. An advantage of the area-based methods compared to the TIM-assay is the large number of cells that can be assessed with a single assay, and the large number of samples that can be processed. Another advantage may be a perhaps more '*in-vivo*-like' situation with a gradient in tumor cell density. Therefore, a comparison of the results obtained with these different assays has to be done with great care.

Using another type of assay, based on artificial basement membrane wafers (Matrigel), Bernstein and coworkers [18] reported for C6 cells an MV of 18.75 μm/h. As Matrigel contains different basement membrane components, it is possible that some of these are permissive for migration and/or proliferation. Again, this study was based on global changes of cell distribution, and the lack of a clear-cut differentiation between proliferation and migration may lead to biased results. This endpoint is also dependent on proteolytic remodeling of the Matrigel to allow cell transit.

There are only three studies on single-cell evaluation of glioma cell cultures. Chicoine and Silbergeld [7] reported with 'time-lapse videomicroscopy' an average cell velocity of 12.5 μm/h for cells that were not further specified. In fact, this result is similar to the mean v_{tumor} of 11.3 μm/h demonstrated in the present study.

Nevertheless, this may be a coincidence as we found tremendous differences of the v_{tumor}, ranging from 0 to 24.2 μm/h for individual cell lines. In addition, an observation time of only 129 min does not seem to be sufficient for reproducible results, due to the great fluctuation of the MV. Hegedüs et al. [8] reported for human glioblastoma cell lines average cell velocities of 4.2 to 27.9 μm/h, which confirms the median velocities found in our study.

With the TIM-assay, being able to analyze proliferation and migration at the same time we observed that proliferating cells stop migration, condense their cytoplasm, divide, expand and continue migration. Basically, all migrating cells showed mitotic activity when observed up to 42 h. The fact that the MI ranged between 78% and 99% demonstrates that there are only few cells, that proliferate but do not migrate. Theoretically, inhibition of cell proliferation by hydroxyurea should result in an increased MV, because of the absence of migration stops during mitosis. However, preliminary results show a decreased MV in cells treated with hydroxyurea, compared to untreated cells using the TIM-assay (data not shown).

The migration velocity (MV) was analyzed by calculating the median velocity for single cells (v_{cell}) and for an individual cell line (v_{tumor}). By comparison of v_{cell} from cells that could be tracked for less than 8 h with cells observable for more than 8 h, significant differences were found. They may result from the fact that cells were either quite close to the borders of the field of observation or cells were migrating very fast, and therefore leaving the field of observation quickly. Since a short period of fast cell movement is present in nearly all cells observed (i.e., last 6 hours for the cell in Figure 2C) we assume, that we would miss phases of slower movement and therefore overestimate MV by not excluding those cells.

The significant differences in v_{tumor} for the various cell lines are thought to reflect interindividual differences for populations of glioblastoma cells *in vitro*.

From Figure 2A one could assume a quite low v_{cell} by just focusing on a 6-h period from hour 4 to 10. On the other hand, when focusing on hour 18 to 24, one could find a much higher v_{cell}. Therefore, we are able to show that prolonged observation times of up to 24 h allow the leveling out of the demonstrated fluctuations of v_{act}. We do not think that the MVs are highly compromised by the human-based procedure of marking cell nuclei by mouse-click. However, we are still working on an automated cell-tracking system. But first trials demonstrated considerable difficulties in reliable recognition of the nuclei. This corresponds with the findings by Hegedüs et al. [8].

We could demonstrate that the majority (61%) of all cells show a random fluctuation in v_{act} over 24 h. The decrease in cell velocity after one day is thought to result from mechanical inhibition of cell movement by an increased cell density. This assumption is supported by a good correlation between the onset of progressive MV deceleration, i.e., 42 h after splitting, and the beginning of the exponential growth phase assessed with standard growth curves. Furthermore, measurements with increased cell density revealed a

decreased MV with the TIM-assay (data not shown). Alternatively, a prolonged assay time may lead to an increase in autologous extracellular matrix secreted by the tumor cells, which facilitates migration [14]. This mechanism seems to play a less prominent role. However, De Hauwer et al. [9] state that an increasing number of cells per colony correlates with increasing cell velocity. This variance may result from differences in the experimental design. De Hauwer et al. performed experiments about 67 h after plating of 6,000 cells per ml, while the TIM-assay starts 24 h after the plating of 3,000 cells per ml. According to the published duplication time of 40 h, De Hauwer et al. start nearly 48 h later with at least 15,000 cells per ml. Since the authors focus their observation on colonies of tumor cells, which may be cores of higher cell density, there could be a gradient in cell density, which allows for a faster movement towards surrounding areas of lower cell density.

On the other hand, there could be a special phase in the life-cycle of a cell about 48 h after splitting (i.e., after 24 h in the TIM-assay) counting for decreasing v_{cell}. Further experiments, carefully leveling out cell proliferation (i.e., using hydroxyurea), need to be done to elucidate the cause of reducing MV with increasing observation time.

Analyzing the morphology for two selected cell lines, we were not able to identify significant correlation of cell morphology and the migratory behavior. However, we were able to identify trends, showing that cells that were tall and slim migrate faster than compact and round ones. No tendency was found for the relationship of MC and cell morphology (data not shown). Further studies need to be done to identify the significance of these findings.

When examining the radial distance migrated for directionally and randomly migrating cells, it was found that directionally migrating cells had a significantly higher radial distance migrated than the random ones as anticipated by the function for a process of diffusion.

For the first time, the individual MP of glioma cells *in vitro* was visualized by plotting all coordinates of a cell at the different points of time. Differentiation between directional and random cell movement was accomplished using the Rayleigh-test. We were able to show that two thirds of all cells showed a directional movement. These results differ from those reported by Chicoine and Silbergeld [17], who described a directional movement for all cells evaluated with the 'radial dish assay'. Since the 'radial dish assay' reflects only global observation of an amount of cells covering a defined area, a detailed analysis of the migration paths is obviously impossible.

In contrast to the findings of Chicoine and Silbergeld, Hegedüs et al. [8] reported random movement of cells over the observation time. We think that this observation also results from the lack of adequate instruments testing for directional or random movement as used in our study. Studies with longer observation times need to be done in order to evaluate the directionality of cell migration over a longer period of time (i.e., 48 to 72 h). One could imagine that even cells, that were characterized as randomly migrating may show directional migration after a certain time.

It is well known that glioma cells follow distinct anatomical structures as myelinated fiber tracts or perivascular space [1, 19] caused by a certain composition of ECM proteins or surface antigens of different cells. The fact that more than 80% of glioma cells from certain cultures exhibit directional movement without guiding structures leads to the question of the biological relevance of this finding. One could imagine that a tumor with a high percentage of directionally migrating cells may be highly invasive. These cells may act as 'pathfinders' being able to leave the tumor core and invade the surrounding brain tissue. Studies based on co-cultures have to elicit this hypothesis. On the other hand, one has to consider that directionality may be an artifact of cell culture. For the TIM-assay, topographical differences in temperature, ECM-proteins and cell-density were ruled out carefully, but nevertheless directional migration occurred. This may be explained by intrinsic factors such as a certain distribution of contractile filaments leading to cell polarity. Such a polarity may be set up and influenced by adhesion mechanisms since actin filaments were found to concentrate at the site of tight junctions [20]. On the other hand, there may be cells migrating over surfaces that have been covered or coated by other cells, that may have been there previously. Therefore, autologous ECM may influence the migratory behavior of other cells. Further experiments have to elicit the influence of ECM on the directionality of glioma migration *in vitro*.

Visual analysis of the MPs of directionally migrating cells revealed three types of migration paths, i.e., oscillating, linear or mixed. Classifications of two raters revealed good interobserver agreement backing up the reliability of the parameter 'pattern of migration path'. A high congruence of cell motility classified as 'linear' by two observers with cluster solutions for the MC was found. Therefore, the MC seems to match the visual impression of 'linear migrating' cells. However, for 'mixed' and 'oscillating' cells there is no good match. This may reflect difficulties in visual classification of 'non-linear migrating' cells. Therefore one could summarize these cells as 'non-linear migrating' cells. At the moment, we do not have any explanation for the discrepancy between the classification by MC (objective) and visual analysis (subjective).

Further studies to find out the visual counterpart of the two additional cluster solutions are under way.

The different patterns of migration paths (linear, oscillating and mixed) may account for differences in the invasive behavior of tumors with a similar percentage of directionally migrating cells. A tumor with a high MV and an oscillating MP, may be less invasive than a tumor with a medium or even low MV but a linear MP because cells of the latter tumor may be found at a greater distance from the original tumor due to their straight MP.

Conclusions

The TIM-assay was introduced to enhance the knowledge on glioma cell migration. The described method was found to be capable of evaluating different migratory aspects of

individual glioma cells *in vitro* under controlled conditions. For the first time, the individual migratory activity of human glioblastoma cells could be analyzed defining by the migration velocity (MV) and the migration path (MP). In addition, the proportion of migrating cells of individual cell lines was determined by a migration index (MI). The MV was found to differ to a great extent for the different glioblastoma cell lines (0–24.2 μm/h, mean 11.7 μm/h) as well as for individual cells of the same culture (Mz18: 0–56.1 μm/h). Directional migration was documented in two thirds of the examined glioblastoma cells. The MP of the individual cells may be further characterized as oscillating, i.e., frequent forward and backward movements, or linear, i.e., straight movements. All cell lines showed a high percentage of migrating cells with a mean MI of 91%. We believe that the described parameters may become an important supplement to the assessment of proliferation parameters and standard histopathological evaluation. For example, a tumor with a high MV and a low MC may be less invasive than a tumor with a medium or even low MV but a medium or high MC, because cells of the latter tumor may be found at a greater distance from the original tumor due to their straight MP (= high MC). Comparison of clinical and histopathological findings are crucial to determine the clinical value of the introduced method and the new parameters.

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