Phosphatidylinositol 3-Kinase in the G Protein-Coupled Receptor–Induced Chemokinesis and Chemotaxis of MDA-MB-468 Breast Carcinoma Cells: A Comparison with Leukocytes

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Abstract
The polarization of tumor cells and leukocytes into a front end and a rear end is a crucial prerequisite for their autonomous, directed movement. Phosphatidylinositol 3-kinase (PI3K) is assumed to play an important role in this polarization process, whereas the results obtained with different cell types and different migration assays widely vary. Thus, we conducted a comparative study on the role of the PI3K in the locomotor activity and directionality of the migration of tumor cells on the example of MDA-MB-468 breast carcinoma cells in comparison with CTLs and neutrophil granulocytes. We used our well-established, collagen-based, three-dimensional migration assay for the investigation of the chemokinesis and chemotaxis of these cells. Our results show that the role of the PI3K in the regulation of migratory activity is distinct between the investigated cell types: the migration of CTLs and MDA-MB-468 cells was impaired by the inhibition of the PI3K with wortmannin, whereas neutrophil granulocytes were only slightly affected. However, neither cell type was impaired in the ability to respond chemotactically to gradients of ligands to G protein-coupled receptors. Thus, the PI3K contributes to the regulation of migratory activity but not to the directionality of migration of MDA-MB-468 breast carcinoma cells. As a further conclusion with regard to cancer treatment, the PI3K is not a suitable target for the inhibition of metastasis formation, because the migration of leukocytes is also affected, which leads to a dysfunction of the immune defense. (Mol Cancer Res 2006;4(6):411–21)

Introduction
Autonomous migration within an adult organism is a very special ability, which is exclusively done by a few cells. Most prominently, these cells are on the one hand the leukocytes, which need to migrate to reach sites of inflammation, or secondary lymph organs and protect the organism against pathogens. On the other hand, there are the tumor cells. They display migratory activity during invasion and metastasis development, which are the ultimate pernicious steps in tumor progression. Because most cancer patients these days die not from the primary tumor but from the formation of metastases, the development of antimetastatic drugs has come into the focus of tumor biologists (1).

The intracellular signal transduction that leads to the generation of locomotory forces is in large parts the same in tumor cells and leukocytes [e.g., with regard to the initiation of migration by ligands to G protein-coupled receptors (GPCR; refs. 2, 3) or the development of locomotory forces by nonmuscle myosin II (4)]. In contrast, certain parts of the regulatory machinery greatly differ between these cells [e.g., the engagement of β2 integrins (5) or certain protein kinase C isotypes (6, 7)]. Such differences might provide future access points to develop strategies for the targeted inhibition of tumor cell migration without side effects on leukocytes or other migrating cells (e.g., stem cells or fibroblasts) and have thus important implications in oncology and immunology.

However, some parts of the regulation of cell migration are yet not fully understood. Surprisingly, one very elementary part, which is still unclear and thus in the focus of current investigations, is the formation and maintenance of polarization of the migrating cells into a leading front end and a rear end. This polarization is the initial step and an inalienable prerequisite for any cellular movement, but especially for the directed movement toward chemoattractive substances, a process known as chemotaxis. The most prominent group of substances that induces chemotaxis is constituted by the ligands to GPCRs [i.e., chemokines and neurotransmitters (3)]. In chemotactic response to these substances, cellular gradients of second messenger molecules, such as calcium (8, 9), diacylglycerol (9), or cyclic AMP (10), have been assumed to be responsible for the initiation and maintenance of polarity. Furthermore, a spatially distinct activation of monomeric GTPases at the front and rear ends may be involved in the polarization of chemotactically migrating cells (11). In addition, there is a growing body of evidence that the phosphatidylinositol 3-kinase (PI3K) plays an important role in the polarization...
process as shown in Dictyostelium discoideum (12). However, current reports on the involvement of the PI3K in other cells are very contrary as has been reviewed by Ward (13). The general hypothesis is that the PI3K is located at the leading front of migrating cells, providing high concentrations of phosphatidylinositol-trisphosphate (PIP3) at these sites by its enzymatic activity (14). PIP3 in turn functions as an adapter molecule for other signaling molecules with a pleckstrin homology domain (15). One of these signaling molecules is the kinase Akt/protein kinase B, which phosphorylates PAKα (16). PAKα is important for the myosin II assembly as shown in the migration of D. discoideum (17). Myosin II is an essential motor protein in the migration of tumor cells and leukocytes as well (4). Furthermore, Akt/protein kinase B phosphorylates the actin-binding protein girdin/APE, which is involved in stress fiber formation and lamellipodia development of Vero cells (18). Thus, Akt mediates effects of the PI3K on the two major cellular motor proteins, actin and myosin.

The PI3K occurs in several subtypes, which are activated by various pathways. Activation is facilitated either by phosphorylation by receptor tyrosine kinases (19), nonreceptor protein tyrosine kinases (PTK) of the src family (20), and focal adhesion kinase (21) or the PI3K is activated by the βγ subunit of activated heterotrimeric G proteins (19). The phosphatases PTEN (22, 23) and SHIP (23, 24) dephosphorylate PIP3 and are thus the antagonists of the PI3K. In a model of lymphocyte chemotaxis, these phosphatases are located at the rear end of migrating cells, thereby amplifying the PIP3 gradient, which is caused by the PI3K at the leading front (13). However, this model does not hold true in general [e.g., it was shown that this localization is not established in migrating HL-60 cells (25)]. In these cells, PTEN had a cytoplasmic distribution.

With regard to the inconsistency of present results on the role of the PI3K in cell migration, which is obviously also due to both use of different cell types and assays for investigation, we herein present a comparative study on the involvement of this kinase in the chemokinesis and chemotaxis of CTLs, neutrophil granulocytes, and tumor cells, the latter on the example of cells of the breast carcinoma cell line MDA-MB-468. Using the same set of analysis methods for every cell type, this work contributes to the understanding of the differential involvement of the PI3K in the undirected (chemokinetic) and directed (chemotactic) movement of these cells.

Results

Involvement of the PI3K in the Migration and Chemotaxis of Leukocytes

Neutrophil granulocytes and CTLs display only minor spontaneous migratory activity after incorporation into a three-dimensional collagen matrix (Fig. 1). However, after treatment with 1 μg/mL stromal cell-derived factor-1 (SDF-1), the CTLs significantly increased their migratory activity from 23.3 ± 20.7% to a maximum (after 15 minutes) of 70.1 ± 27.2% locomoting cells (P = 0.034; Fig. 1A). After treatment with 10 nmol/L formyl-methionyl-leucyl-phenylalanine (fMLP), the neutrophil granulocytes significantly increased their migratory activity from 2.5 ± 2.8% to 83.1 ± 2.6% locomoting cells (P < 0.001; Fig. 1B). These two types of leukocytes show differences in response to the treatment with 250 nmol/L of the PI3K inhibitor wortmannin. The spontaneous migratory activity of CTLs was reduced to 3.9 ± 3.6% locomoting cells, which is, however, not significant (P = 0.11), and the SDF-1-induced migration is significantly reduced to 11.5 ± 8.0% locomoting cells after 1 hour (P = 0.029; Fig. 1A). In contrast, the migration of neutrophil granulocytes was only marginally influenced: the migratory activity is 2.5 ± 3.0% locomoting cells after treatment with wortmannin and 70.9 ± 14.2% locomoting cells after treatment with fMLP and wortmannin in combination (Fig. 1B). Higher concentrations of wortmannin (1 μmol/L) did not increase the observed effects, whereas lower concentrations had less effects (data not shown). Thus, 250 nmol/L was the minimum concentration with optimum effect. None of the used concentrations affected the viability of the cells as assessed by propidium iodide staining and flow cytometry.

The velocity of migration remained unchanged after treatment with wortmannin in both cell types (Fig. 1C), whereas the respective GPCR ligands induced an increase of the velocities, in accordance with results which we have published previously (26). With regard to the experiments shown herein, the stimulation by SDF-1 was not significant in CTLs, but fMLP significantly (P < 0.01) increased the velocity in neutrophil granulocytes.

Similar to the inhibition of the PI3K, its activation had different effects in CTLs and neutrophil granulocytes (Fig. 2). In contrast to the inhibition experiments, activation of the PI3K using the pharmacologic activator 740-Y-P had no effect in CTLs (Fig. 2A) but led to a slight, dose-dependent increase of the migratory activity in neutrophil granulocytes (Fig. 2B).

Both cell types migrate chemotactically toward the source of the GPCR ligands (Fig. 3). After application of SDF-1, 84.4 ± 6.4% of the CTLs migrated toward the source (Fig. 3A), and after application of fMLP, 82.6 ± 9.2% of the neutrophil granulocytes migrated toward the source (Fig. 3B). Treatment with 250 nmol/L wortmannin did not influence the chemotactic response in either case (Fig. 3C and D). Here, 77.2 ± 13.5% of the CTLs and 90.0 ± 4.7% of the neutrophil granulocytes migrated toward the source. All of these chemotactic responses were statistically significant (all P < 0.001, with the exception of CTLs treated with SDF-1 and wortmannin, which was P = 0.0012).

Involvement of the PI3K in the Migration and Chemotaxis of MDA-MB-468 Breast Carcinoma Cells

In contrast to leukocytes, MDA-MB-468 breast carcinoma cells develop high spontaneous migratory activity after incorporation into the collagen matrix (35.2 ± 10.5% migrating cells; Fig. 4A). In addition, norepinephrine induced a significant increase of the migratory activity to 51.4 ± 11.2% migrating cells (P = 0.046; Fig. 4A). This increase was abolished by the inhibition of the PI3K with 250 nmol/L wortmannin (33.8 ± 14.3% migrating cells), whereas the spontaneous locomotor activity was not affected (33.8 ± 17.5% migrating cells). Even 1 μmol/L wortmannin had no influence on the spontaneous migratory activity (data not shown).

As we have investigated previously (27), the MDA-MB-468 cells chemotactically respond to norepinephrine (Fig. 4B); here, 55.4 ± 7.6% of the tumor cells migrated into the top left quadrant toward the source of norepinephrine, 11.2 ± 5.9% of

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the cells migrated toward the top right quadrant, $22.1 \pm 7.6\%$ of the cells migrated toward the bottom left quadrant, and $11.2 \pm 5.3\%$ of the cells migrated away from the source to the bottom right quadrant (top left quadrant versus each other quadrant; $P < 0.001$). After additional treatment with wortmannin (250 nmol/L), the migration paths were shorter, in accordance with the reduced migratory activity shown in Fig. 4A. However, still $55.1 \pm 10.7\%$ of the tumor cells migrated into the top left quadrant toward the source of norepinephrine, $20.5 \pm 8.7\%$ of the cells migrated toward the top right quadrant, $10.4 \pm 7.5\%$ of the cells migrated toward the bottom left quadrant, and $13.8 \pm 7.4\%$ of the cells migrated away from the source to the bottom right quadrant (top left quadrant versus each other quadrant; $P < 0.001$).

**Localization of the PI3K, SHIP, and PTEN in Cells Migrating within a Three-Dimensional Collagen Matrix**

In CTLs, the PI3K has been described to be located at the leading front of migrating cells, and the antagonistic phosphatase PTEN is located at the rear end; the second

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**FIGURE 1.** Inhibition of the PI3K in the migration of CTLs and neutrophil granulocytes. **A.** Migration of CTLs was stimulated using 1 μg/mL SDF-1; the PI3K was inhibited with 250 nmol/L wortmannin. Left, time course; right, migratory activity after 1 hour. Columns, mean of four independent experiments (120 cells were analyzed per sample); bars, SD. Con, control; wort, wortmannin; SDF+W, SDF-1 + wortmannin. **B.** Migration of neutrophil granulocytes was stimulated with 10 nmol/L fMLP; the PI3K was inhibited with 250 nmol/L wortmannin. Left, time course; right, migratory activity at the steady-state level. Columns, mean of three independent experiments (90 cells were analyzed per sample); bars, SD. NG, neutrophil granulocytes; fM, fMLP, fM+w, fMLP + wortmannin. **C.** Velocities were calculated of the same cells as in **A** and **B**. *, $P < 0.05$, statistically significant changes. W, wortmannin; fMLP+W, fMLP + wortmannin.
phosphatase SHIP is supposed to be at the rear end as well. This spatially distinct distribution is supposed to keep the PIP3 gradient stable (13). We have confirmed this distribution in CTLs in our three-dimensional migration system (data not shown) and have furthermore found the same distribution of these molecules in migrating neutrophil granulocytes on stimulation with fMLP (Fig. 5). The fMLP receptor had a scattered distribution on the whole cell, however, with a preference for the leading area (Fig. 5A). Accordingly, the PI3K was located at the front of the migrating cell (Fig. 5B), and the phosphatases SHIP and PTEN were located at the rear end of the cells or were at least excluded from the front (Fig. 5C and D). Nonmuscle myosin II, which has an important role for the migratory activity of the neutrophil granulocytes (4), was colocalized with F-actin (Fig. 5E). F-actin accumulation had a bipolar distribution: it was either located at the front (Fig. 5D and E), at the end (Fig. 5A and B), or at both sites of the cells at the same time (Fig. 5C) as reported previously by Eddy et al. (28). We assume that these differences in the actin distribution reflect different states in the migration cycle.

The promigratory effect of norepinephrine on MDA-MB-468 breast carcinoma cells is mediated by the \( \beta_2 \)-adrenoceptor (27). As we have published previously (29), the \( \beta_2 \)-adrenoceptor was predominantly located at the leading front in these migrating cells (Fig. 6A). At this part of the cells, most of the F-actin was found as well (Fig. 6, bottom row), where it was colocalized with nonmuscle myosin II (Fig. 6E). In contrast, neither the PI3K nor the phosphatases SHIP and PTEN showed a perceptibly polarized distribution within the migrating tumor cells (Fig. 6B-D).

**Activation and Localization of Akt**

Akt/protein kinase B is one of the key target molecules of the PI3K. Akt is phosphorylated by the phosphoinositide-dependent kinase-1, which is activated on PIP3 binding (30). In turn, Akt/protein kinase B has several substrates, which are involved in the regulation of cell migration. We have investigated the phosphorylation of Akt and the localization of this activated form in the migrating cells. Akt is phosphorylated in each cell type on stimulation with the respective ligand (SDF-1, fMLP, or norepinephrine; Fig. 7A-C). Unstimulated CTLs had no detectable amounts of phosphorylated Akt (p-Akt; Fig. 7A), and in neutrophil granulocytes, only a very weak staining was detectable (Fig. 7B). In both leukocytes, treatment with the respective stimulus strongly increased the phosphorylation of Akt, whereas this phosphorylation was prevented by wortmannin treatment. Because of the lack of p-Akt in the control, quantification and comparative statistical analysis in leukocytes was not meaningful. In MDA cells, Akt was already strongly phosphorylated without additional stimulation (Fig. 7C). Nevertheless, stimulation with norepinephrine led to an increase of the phosphorylation by 124%, whereas wortmannin reduced the phosphorylation to...
10%. Treatment with both substances led to a reduction to 23% of the control (Fig. 7C). The localization of p-Akt was in each cell type similar to the localization of the PI3K (Fig. 7D-F). Only confocal images of stimulated cells as shown, because unstimulated leukocytes had no detectable amounts of p-Akt. In leukocytes, p-Akt staining occurred predominantly in the leading part of the cells, whereas the overall staining especially in CTLs was very low (Fig. 7D and E). In MDA cells, p-Akt staining occurred at those areas, which were high for F-actin (Fig. 7F).

Discussion

The three-dimensional collagen-based migration assay is probably the in vitro migration assay, which is closest to the in vivo situation (31). Hence, some observations made by the use of other migration assays (especially two-dimensional surface assays) do not hold true in this assay [e.g., with regard to the development of actin stress fibers (4), the assembly of focal adhesion contacts (32, 33), or the engagement of integrins (34)]. Likewise, the inhibition of the PI3K did not impair the chemotactic response in CTLs, neutrophil granulocytes, or tumor cells in our assay. This is in accordance with Ward’s line of argumentation that at least in CTLs the “PI3K activation can be a dispensable signal for directed cell migration in certain settings” (13). It is obvious that various migration assays lead to different results with regard to the role of the PI3K in cell migration and chemotaxis. In contrast to our observations, PI3K-negative neutrophil granulocytes, which adhere to glass coverslips, mostly failed to perform chemotaxis toward fMLP as shown by the use of a Zigmond chamber (35). Furthermore, differences might occur from different cells investigated [e.g., the PI3K has been proven to play a crucial role in the chemotaxis of D. discoideum (12, 36)]. In contrast, in tumor cells, PI3K-independent mechanisms can also regulate directed migration as summarized by Huttenlocher (37).

In our assay, the PI3K is differentially involved in the regulation of the migration of CTLs, neutrophil granulocytes, or breast carcinoma cells, but, as discussed above, the PI3K does not contribute to the directionality of migration in either cell type. As introduced above, the current model on the involvement of the PI3K in the chemotaxis is based on the spatially distinct distribution of this kinase and its antagonistic phosphatases within the cells. Therefore, we investigated the distribution of these molecules by confocal laser scanning microscopy. We have found a polarized distribution of the PI3K and its antagonistic phosphatases SHIP and PTEN in the investigated leukocytes but not in tumor cells. Thus, leukocytes are potentially able to develop a PIP3 gradient from the differential distribution of these molecules, whereas this is not possible in tumor cells.

The involvement of the PI3K in the regulation of spontaneous and induced migration of these three cell types is similar to the regulation by srcPTKs (Table 1). In CTLs, both types of migration were strongly PTK dependent, whereas inhibition of these kinases only slightly influenced the fMLP-induced migration of neutrophil granulocytes (26). In tumor cells, only the GPCR-induced migration is PTK dependent (38). Thus, it is likely that srcPTKs are the upstream activators of PI3K in the signal transduction of migration. Downstream, nonmuscle myosin II is an important molecule for the generation of locomotor forces (4). Likewise, both types of CTL migration (spontaneous and SDF-1 induced) are nonmuscle

![Figure 3](image-url)
myosin II dependent, and only the norepinephrine-induced migration of MDA-MB-468 breast carcinoma cells is non-muscle myosin II dependent (4), which is in correlation to the herein discovered involvement of the PI3K. In contrast, the migration of neutrophil granulocytes strongly depends on nonmuscle myosin II as well, which suggest a distinct regulation of migration in these cells (Table 1). As discussed in Introduction, Akt/protein kinase B phosphorylates PAKa, which plays a role in myosin II assembly (17), and has influence on the actin stress fiber development (18). The PI3K/Akt pathway is frequently altered in human cancers (30), and an activation of the phosphoinositide-dependent kinase-1 and Akt is correlated with invasive and metastasizing breast tumors (39). Accordingly, Akt is already phosphorylated in the tumor cells used herein without a stimulus, whereas it is not phosphorylated in the leukocytes. In all three cell types, an increase of the Akt phosphorylation occurred after stimulation of migration, whereas wortmannin effectively inhibited this phosphorylation. It is thus likely that Akt is a downstream mediator of the PI3K for the stimulation of migratory activity.

With regard to the induced type of migration, one has to consider the argument that different ligands were used for the three investigated cell types. Although all receptors to these ligands were GPCRs, the intracellular signal transduction might vary, because the α-subunits of the engaged G proteins have different functions. The α-subunits of Gq proteins stimulate the adenylyl cyclase, and the α-subunits of Gi proteins have an inhibitory function on this cyclase (40). The α-subunits of Gs proteins activate the phospholipase Cβ1 (41). It is generally accepted that the SDF-1 receptor is predominantly coupled to Gq proteins (42), although there are some reports that this receptor also couples to Gi proteins (43). The fMLP receptor is Gi coupled (44). In MDA-MB-468 breast carcinoma cells, the β2-adrenoceptor is responsible for the initiation of migration (27). This receptor is coupled to Gi proteins (42, 43). Interestingly, norepinephrine has stimulatory effects in CTLs (45) but inhibits the fMLP-induced migration of neutrophil granulocytes (Fig. 8). Thus, in neutrophil granulocytes, Gi-coupled GPCRs stimulate the migration and Gs-coupled receptors are inhibitory. This deviation to CTLs and tumor cells suggests further differences in the signal transduction of neutrophil granulocytes. Because PTKs are activated by the γ-subunit of the heterotrimeric G proteins via GPCR kinases and β-arrestin (46, 47), this PI3K-activating signal transduction pathway is likely to be the same among the different cell types. Thus, differences in the effect of various GPCR ligands should be due to a differential involvement and function of the pathway that is initiated by the α-subunit of the heterotrimeric G proteins.

In summary, the PI3K is involved in the regulation of migratory activity in tumor cells and leukocytes in a varying manner. However, the PI3K does not contribute to the chemotactic movement in any of the investigated cell types; alternatively, its inhibition can be compensated by other pathways. Although we found a polarized distribution in CTLs and neutrophil granulocytes, PI3K at the leading front and SHIP/PTEN at the rear end, there is no evidence for the necessity of a PIP3 gradient for the directed movement in these cells.
cells. With respect to the involvement of the PI3K in cell migration in general, the chemokinetic response (migratory activity) seems to be independent from the chemotactic response (directionality of migration), which in some migration assays is difficult to analyze separately (31). In conclusion, if PIP_3 is not the (sole) second messenger to maintain the polarization in these chemotactically migrating cells, what else could this be? From our own previous experiments, we hypothesize that calcium is involved in the initiation and maintenance of migration at least in neutrophil granulocytes (8) and tumor cells (48). However, further investigations on this need to be conducted, and as described in Introduction, several other molecules or pathways are still candidates for the initiation and maintenance of polarization, which is likely to vary among different cells.

Our data show that the GPCR-induced migration of tumor cells can be prevented by the inhibition of the PI3K but only with severe inhibitory side effects on the migration of leukocytes. In a broader context, the PI3K seems to be an essential regulatory molecule, whose inhibition would not only concern tumor cells. Furthermore, the PI3K plays an important role in the drug resistance of leukemia cells (49). The authors of this study recommend that further investigations on the involvement of certain PI3K isotypes need to be done, before a clinical use pharmacologic inhibitors in oncology can be estimated. A clinical application of such inhibitors is of course only useful if the active PI3K isotype in tumor cells is dispensable in all other cells of the organism or its inhibition can be compensated in these cells.
Materials and Methods

Cell Isolation and Cell Culture

CTLs and neutrophil granulocytes were isolated from human peripheral blood in two steps (26). First, the mononuclear cell fraction was separated from the erythrocyte/granulocyte fraction by density-gradient centrifugation using Lymphocyte Separation Medium (PAA, Pasching, Germany). Subsequently, CTLs were isolated from the mononuclear fraction by a positive selection using immunomagnetic beads coated with mouse anti-human CD8 antibody (Dynabeads, Dynal, Hamburg, Germany). The cell-bound beads were detached with polyclonal anti-mouse F(ab) fragments (Detachabead, Dynal). Isolated CTLs were maintained overnight in RPMI (PAA), 10% heat-inactivated FCS (PAA), and 1% penicillin/streptomycin (50 units/mL and 50 μg/mL; Life Technologies, Eggenstein-Leopoldshafen, Germany) at 37°C in a humidified atmosphere containing 5% CO₂.

The pellet of the density-gradient centrifugation containing the erythrocyte/neutrophil granulocyte fraction was mixed with platelet-depleted serum of the same blood donor and subsequently diluted 1:1.3 with a high molecular weight dextran solution (Macrodex, Fresenius, Bad Homburg, Germany) containing 0.01 mol/L EDTA. After 1.5 hours, the supernatant containing the neutrophil granulocytes was separated from the pellet containing the erythrocytes. Remaining erythrocytes were removed by a hypotonic lysis with 0.3% NaCl for 3 minutes on ice. The purified neutrophil granulocytes were used immediately after isolation.

The human breast carcinoma cell line MDA-MB-468 (American Type Culture Collection, Rockville, MD) was cultured in Leibovitz’s L-15 culture medium (PAA) containing 10% heat-inactivated FCS and kept at 37°C in a humidified atmosphere.

Cell Migration Assay and Data Analysis

The migratory activity of the cells within three-dimensional collagen lattices was recorded by time-lapse videomicroscopy and analyzed by computer-assisted cell tracking as described in Table 1.

Table 1. Involvement of Signal Transduction Components in the Migration of Leukocytes and Tumor Cells

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FIGURE 7. Phosphorylation of Akt and its localization in migrating cells. A to C. Immunoblots of whole Akt and p-Akt after stimulation of the cells with the according stimulus and inhibition with wortmannin. S+W, SDF-1+wortmannin. D to F. Localization of p-Akt within stimulated migrating cells (top row) compared with F-actin staining (bottom row). White arrows, direction of migration.
previously (4). In brief, \(2 \times 10^5\) leukocytes or \(8 \times 10^5\) tumor cells were mixed with 150 µL carbonate-buffered liquid collagen (Flow, McLean, VA) containing Eagle’s MEM (Sigma-Aldrich, Taukirchen, Germany) as well as the stimulators for migration and either the PI3K inhibitor wortmannin (250 nmol/L; Calbiochem, Bad Soden, Germany) or the PI3K activator 740 Y-P (1-100 µmol/L; Biotrend, Cologne, Germany). To stimulate the migratory activity, we used for each cell type the most potent inducer of migration known. These were the SDF-1 (1 µg/mL; Biotrend) for CTLs, fMLP (10 nmol/L, Sigma-Aldrich) for neutrophil granulocytes, and norepinephrine (10 µmol/L; Sigma-Aldrich) for the tumor cells. The cell-collagen mixtures were filled into self-constructed migration chambers, which consisted of a glass slide at the bottom, wax walls on the sides, and a coverslip on top (4). The collagen suspensions were then allowed to polymerize for 30 minutes at 37°C.

Chemotaxis was investigated in a collagen-based migration system equal to the undirected (chemokinetic) migration as described above, with the difference that special chemotaxis chambers were used. In these chambers, the migration-stimulating substances were applied through a capillary from one side. More details on the chemotaxis chambers and the method are described in the references (26, 27, 31, 50).

In either case (undirected, chemokinetic migration, or chemotaxis), after recording, the migration paths of 30 randomly selected cells were digitized in 1-minute intervals (leukocytes) or 15-minute intervals (tumor cells). The migratory activity was calculated for each step as the part of the cells (in percent), which was locomotory active. All migratory activities shown in the figures are the mean of at least three independent experiments (for the leukocyte experiments, the cells of different blood donors were used); in the text, we provide the mean and SD at the steady-state level of the observation period.

The velocity (µm/min) was calculated as the average of the mean single-cell velocities at the periods of actual movement (excluding pauses). Statistical significance of changes was calculated using the Student’s t test.

Directionality was analyzed by the following procedure: the net movement of each single cell was calculated by the direct connection of the start and end points of each individual path.

Confluent Laser Scanning Microscopy

To analyze the intracellular distribution of Akt and p-Akt, the PI3K, PTEN, and SHIP in combination with F-actin, and the distribution of the respective receptors for the migration-stimulating substances (β2-adrenoceptor and fMLP receptor) on the surface of the cells, collagen lattices containing tumor cells and neutrophil granulocytes were generated as described in Cell Migration Assay and Data Analysis. Samples were treated with 4% paraformaldehyde overnight at 4°C for fixation, washed, and subsequently incubated with 0.5% Triton X-100 for 10 minutes at 20°C for permeabilization of the cells. After a further washing step, the samples were incubated for 1.5 hours at 20°C with an anti-PI3K, anti-PTEN, or anti-SHIP antibody. All of these antibodies were mouse monoclonal IgG antibodies derived from Becton Dickinson (Heidelberg, Germany). Akt and p-Akt were stained with rabbit monoclonal IgG antibodies (New England Biolabs, Frankfurt am Main, Germany). After washing, the samples were incubated for 1.5 hours at 20°C with a FITC-conjugated secondary goat anti-mouse F(ab) fragment (Jackson Immunoresearch, Baltimore, PA) or a FITC-conjugated goat anti-rabbit F(ab) fragment (Southern Biotech, Birmingham, AL). The fMLP receptor was stained on neutrophil granulocytes with a mouse anti-fMLP receptor IgG antibody (Becton Dickinson), and the same secondary F(ab) fragment that was used for the PI3K/PTEN/SHIP staining. The β2-adrenoceptor was stained on the MDA-MB-468 cells with a rabbit polyclonal anti-β2-adrenoceptor IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and with the same FITC-conjugated goat anti-rabbit F(ab) fragment that was used for Akt/p-Akt staining. F-actin was stained using Alexa Fluor-conjugated phalloidin (Molecular Probes, Leiden, the Netherlands). The staining procedure was closed by intensive washing of the samples.

Confocal laser scanning microscopy was done using a TCS-4D microscope (Leica, Bensheim, Germany) as described previously (6). Both FITC and Alexa Fluor fluorescence were...
detected in parallel together with the transmission light to visualize the morphology of the cells. The confocal reflection contrast was used to visualize the collagen fibers. The cells were scanned in the Z axis by 1-μm intervals. Fluorescence and reflection contrast images were processed by an overlay of all Z scans.

Immunoblotting

The phosphorylation of Akt was analyzed by immunoblotting with the same primary Akt/p-Akt antibodies that were used for confocal microscopy. Cells were lysed in Laemmli sample buffer (10 minutes at 95 °C), and lysates of 5 × 10^4 tumor cells or 1 × 10^4 leukocytes were applied to gel electrophoresis according to Laemmli (51). The proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) according to Towbin et al. (52). Subsequently, the membranes were blocked with a 5% (w/v) dry milk powder solution in washing buffer (Tris + 0.1% Tween 20) for 1 hour at 20°C. After washing with washing buffer, the membranes were incubated with the p-Akt antibody overnight at 4°C, washed again, and incubated with a horseradish peroxidase–conjugated goat anti-rabbit F(ab) fragment (Southern Biotech) for 1 hour at 20°C. After detection of the luminescence signal using a Hamamatsu C4742-98 system (Hamamatsu, Herrsching, Germany), the membranes were stripped [0.2 mol/L glycine (pH 2.2), 0.1% SDS, 1% Tween 20] for 2 hours at 20°C and reprobed with the Akt antibody by the same procedure. Staining signals of p-Akt were quantified using the ImageJ software (NIH, Bethesda, MD) and adjusted for semiquantification to whole Akt staining.

Acknowledgments

We thank Beate Mainusch for excellent technical assistance.

References

Molecular Cancer Research

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