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Basic fibroblast growth factor released by single, isolated cells stimulates their migration in an autocrine manner

(cell migration/autocrine stimulation/secretion)

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ABSTRACT **Basic fibroblast growth factor (bFGF), a protein with angiogenic, mitogenic, and chemotactic properties, lacks a signal sequence and is not secreted via the classical secretory pathway. However, the growth factor is known to act extracellularly. Since no defined mechanism for bFGF release has been described, it has been suggested that this growth factor is released from dead or damaged cells. To test this hypothesis we characterized the effect of exogenously added bFGF and neutralizing antibody on the migration of single, isolated NIH 3T3 cells transfected with bFGF cDNA. Under these conditions the observed cell cannot be affected by bFGF derived from other cells. Cells were seeded onto colloidal gold-coated coverslips at a density of one cell per coverslip. A cell migrating on this substrate produces a track free of refringent gold particles that is measured by an image analyzer. The results showed that cell motility directly correlated with the amount of bFGF released from the migrating cells. Affinity-purified anti-bFGF antibody, but not irrelevant IgG, reduced the level of migration of the bFGF transfectants to that of the control cells transfected with the vector alone, showing that bFGF stimulates migration of the cell that releases it. Thus, bFGF is secreted by viable cells and mediates cell functions via a "true" autocrine mechanism.**

Basic fibroblast growth factor (bFGF) is the prototype member of a family of structurally related polypeptides that modulate functions of cells of mesodermal, endodermal, and ectodermal origin (1). bFGF is present in virtually all tissues (2, 3) and has various effects on a number of cell types (4–6). It acts as a mitogen for some cells—e.g., fibroblasts—and induces differentiation in others—e.g., neural cells (7, 8). In vascular endothelial cells bFGF stimulates a number of functions involved in the formation of blood vessels (angiogenesis); these include cell proliferation, migration, protease production, and invasiveness (5, 6, 9–11). bFGF is one of the more potent angiogenesis inducers *in vivo* and *in vitro* (12, 13).

bFGF is found associated with heparan sulfate proteoglycans *in vitro* (14–18) and is present in basement membranes *in vivo* (19, 20). Moreover, plasma membrane receptors for bFGF have been identified on a number of cell types (15, 21–24). These findings point to an extracellular mechanism of action of bFGF. In several cell types the number of receptors per cell appears to be inversely proportional to the intracellular content of bFGF (15, 25). This apparent down-regulation of cell receptors has been interpreted as a result of an autocrine mechanism of action. This hypothesis is also supported by the observation that neutralizing antibodies to bFGF alter several properties of bFGF-producing cells, including morphology, growth in soft agar, plasminogen activator synthesis, and cell migration (26, 27). Protamine sulfate and suramin, which block bFGF binding to its receptor(s), have the same effect (25, 28).

These findings are most intriguing because bFGF, as well as acidic FGF, platelet-derived endothelial cell growth factor, ciliary neurotrophic factor, and interleukin 1β, lack a hydrophobic signal sequence that can direct their secretion via the endoplasmic reticulum–Golgi system (29–31). In fact, the 18-kDa form of bFGF appears to be localized primarily within the cytosol (3, 32, 33). Very little or no bFGF is found in the conditioned medium of most cells (34).

Since no defined mechanism for bFGF secretion has been proposed, it has been speculated that this growth factor is released from dead or damaged cells. McNeil *et al.* (35) have shown that mechanically induced disruptions of the plasma membrane can be a significant route of bFGF release from cultured endothelial cells. However, whether this is the only physiological mechanism of bFGF release still remains to be determined. Recently, interleukin 1β has been shown to be secreted by activated human macrophages via a secretion pathway independent of the classical ER–Golgi route (36). In this study interleukin 1β secretion was monitored by immunoprecipitation of the mature cytokine from cell-conditioned medium. This approach cannot be used for studying bFGF release from cultured cells for, unlike interleukin 1, the amount of bFGF present in culture fluids is extremely low and, most often, cannot be detected by conventional methods. More important, under mass culture conditions contamination by bFGF derived from dead or damaged cells cannot be excluded.

To overcome this problem we have devised an experimental system to study the migration of single, isolated bFGF-transfected NIH 3T3 cells. Under these experimental conditions cell migration could only be modulated by bFGF derived from the cell being observed, with no interference by factors derived from lysed or injured cells. The results show that the bFGF exported into the extracellular milieu stimulates migration of the same cell that secretes it via a "true" autocrine mechanism.

MATERIALS AND METHODS

Materials. Gold chloride ($HAuCl_4$) was purchased from J. T. Baker. Recombinant FGF (rbFGF) was a generous gift from Synergen (Boulder, CO). Suramin was purchased from Mobay Chemical.

Cells and Culture Conditions. The clones B1 and B3 of NIH 3T3 cells transfected with the retroviral vector pZip-NeoSV(X) containing a 1.1-kilobase (kb) insert of human

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Abbreviations: bFGF, basic fibroblast growth factor; rbFGF, recombinant bFGF; B1, NIH 3T3 cells transfected with human bFGF cDNA, clone pZipbFGF B1; B3, NIH 3T3 cells transfected with human bFGF cDNA, clone pZipbFGF B3; Zip, NIH 3T3 cells transfected with the viral vector pZipNeoSV(X); BCE, bovine capillary endothelial.

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bFGF cDNA have been described (37). The cells were grown in modified Eagle's medium (DMEM; GIBCO) containing 10% donor calf serum (Flow Laboratories) and 500 μg of G418 per ml (GIBCO) (growth medium). The cultures were split 1:4 every fourth day, before confluency was reached. Bovine capillary endothelial (BCE) cells were isolated from the adrenal cortices of yearling calves as described (38) and grown in gelatin-coated Petri dishes in alpha minimum essential medium (GIBCO) supplemented with 5% calf serum.

Phagokinetic Track Assay. Twelve-millimeter (diameter) microscope coverslips were coated with colloidal gold as described (39) and placed into the wells of 24-well microculture plates (Costar). For phagokinetic track assays, subconfluent cultures were trypsinized and resuspended in growth medium. After counting the cells with a hemocytometer, the cell suspension was diluted to a concentration of one cell per ml of growth medium. One milliliter of the cell suspension was dispensed into the culture wells containing the colloidal gold-coated coverslips. One hour later the medium was changed in order to remove potential contaminating bFGF derived from cells damaged during trypsinization and to eliminate damaged cells that would not promptly adhere to the substrate, since these might leak bFGF. After 16 hr of incubation at 37°C (a time shorter than the cell cycle) the culture medium was removed and 3.7% (vol/vol) formaldehyde was added into the culture wells. The coverslips were stored in 3.7% formaldehyde until they were placed onto microscope slides and phagokinetic tracks were observed using dark-field illumination with a 100× magnification. Track areas were measured by an Artek model 982 image analyzer (Farmingdale, NY) connected to the microscope. The area units obtained by the image analyzer (pixels) were converted into μm^2 by interpolation with a calibration curve obtained by measuring the squares of different size of a hemocytometer. The data of the phagokinetic track assays were elaborated with statistical software (KALEIDAGRAPH, Abelbeck Software; and STATWORKS, Cricket Software, Philadelphia) run on a Macintosh II× computer.

Suramin Treatment of Cells. B1 cells grown in 10-cm Petri dishes were washed twice with 10 ml of phosphate-buffered saline (PBS) with or without 1 mM suramin. The cells were trypsinized and resuspended in 10 ml of growth medium with or without the addition of 1 mM suramin. After centrifugation, the cells were resuspended in growth medium with or without 1 mM suramin and counted with a hemocytometer. The cell suspension was diluted to one cell per ml and the cells were seeded onto gold-coated coverslips in suramin-free medium.

Purification and Characterization of Neutralizing Anti-bFGF Antibody. Rabbit anti-human rbFGF IgG was precipitated from serum (40, 41) with 50% saturated ammonium sulfate and purified sequentially by protein A-Sepharose and rbFGF-Affi-Gel affinity chromatography (41). Affinity-purified and flow-through IgG from the rbFGF-Affi-Gel column were tested for their capacity to neutralize the plasminogen activator-inducing activity of rbFGF on BCE cells as described by Gross et al. (42).

RESULTS

Two clones of NIH 3T3 cells transfected with human bFGF cDNA, pZipbFGF (B1) and pZipbFGF B3 (B3), which express different amounts of bFGF, as well as control NIH 3T3 cells transfected with the viral vector pZipNeoSV(X) alone (Zip), were tested for their ability to migrate on colloidal gold-coated coverslips as described in *Materials and Methods*. The level of bFGF produced by the B1 clone is comparable to that produced by BCE cells and by the human hepatoma cell line SK-Hep1. The B3 clone expresses at least 10 times more bFGF than the B1 clone. The B1 and B3 clones produce the 18-kDa, 22-kDa, and 24-kDa forms of bFGF. All three forms can be detected in the medium of B3 cells (ref. 37; N. Quarto, personal communication). The control Zip cells produce undetectable levels of bFGF (37). Cells of the three clones were seeded onto colloidal gold-coated coverslips at a concentration of one cell per coverslip and observed microscopically after 16 hr of incubation, before cell division occurred. As was expected on the basis of a Poisson distribution with a mean of one cell per well, of the 96 wells into which the cells had been seeded, 20–38 were found to contain one single cell. Two or three coverslips had two cells, whereas the remaining coverslips had no cells. Thus, migration of single, isolated cells could be studied under conditions in which the presence of contaminating cells could be excluded.

Under dark-field illumination the gold particles appear as a homogeneous layer of highly refringent particles on a dark background. A cell migrating on this substrate (Fig. 1) phagocytizes or pushes aside the gold particles, thus producing a dark track free of refringent particles. The migrating cell is visible inside the track as a highly refringent body (39). As shown in Fig. 1, the phagokinetic tracks produced by the control Zip cells appeared smaller than the tracks produced by the (B1 and B3) cells of the two bFGF transfectants. To quantitate these differences the area of the tracks was measured by an image analyzer. The mean track area (±SEM) of the Zip cells was 853.3 ± 66.7 μm^2; those of the B1 and B3 cells were 3932.7 ± 164.2 and 6670.4 ± 573.7 μm^2, respectively (Fig. 2). The differences between the three clones were statistically significant ($P < 0.001$), as evaluated by the Student t and Wilcoxon tests. Thus, B1 and B3 cells of the two bFGF transfectants were significantly more motile than the control Zip cells. Moreover, the B3 cells appeared to be more active in migration than the B1 cells, which express a lower level of bFGF. These results suggested that the motility of these cells directly correlated with the amount of bFGF they produced.

The effect of bFGF could be mediated by three different mechanisms: (*i*) bFGF released from cells damaged before or during trypsinization might remain bound to viable cells, (*ii*)

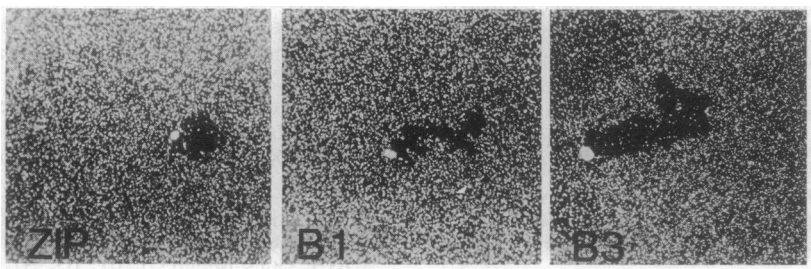

FIG. 1. Phagokinetic tracks formed by single Zip, B1, and B3 cells. Zip, B1, and B3 cells were seeded onto colloidal gold-coated coverslips at the density of one cell per coverslip. After 16 hr of incubation at 37°C the coverslips were fixed with 3.7% (vol/vol) formaldehyde and mounted on microscope slides. Photographs were taken in dark-field illumination with a ×100 magnification. (Final magnification, ×75.)

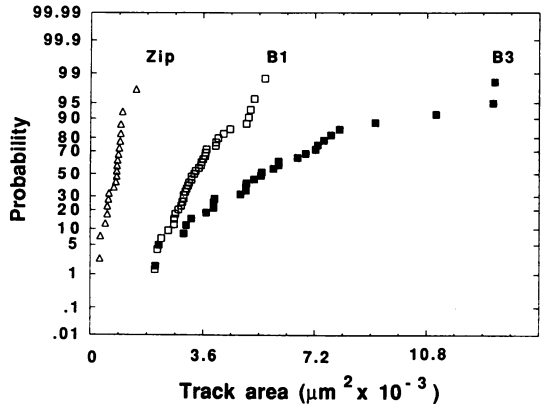

FIG. 2. Distribution of cumulative frequencies of the phagokinetic track areas formed by single Zip, B1, and B3 cells. Single-cell phagokinetic tract assays and elaboration of the data were done as described in the text. Each point on the graph shows the probability of finding a phagokinetic track with an area equal to or lower than the value indicated on the abscissa. The data were plotted using a probability scale in the ordinate. △, Zip cells; □, B1 cells; ■, B3 cells.

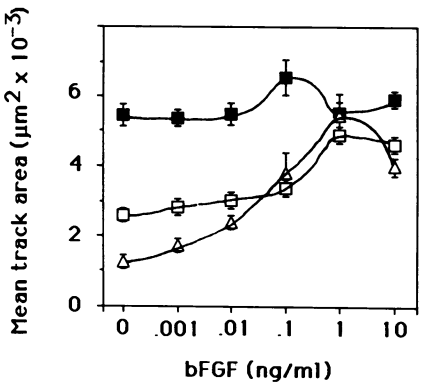

FIG. 3. Dose-dependent stimulation of cell migration by rbFGF. Zip, B1, and B3 cells were seeded onto colloidal gold-coated coverslips at the concentration of 20 cells per coverslip. The indicated concentrations of rbFGF were added in a volume of 10 μl of culture medium at 1 hr after seeding the cells. After incubation at 37°C for 16 hr, all track areas were measured. Each point and relative bar indicate mean track area ± SE determined on 20–40 measurements per sample. △, Zip cells; □, B1 cells; ■, B3 cells.

endogenous bFGF might stimulate single cell motility by acting intracellularly, and (*iii*) bFGF might be released by the migrating cell and interact with its membrane receptor(s). To test the first hypothesis, B1 cells were washed repeatedly with PBS with or without 1 mM suramin prior to seeding, as described in *Materials and Methods*. Suramin has been shown to remove bFGF from the cell surface (28). The mean track area of the suramin-treated cells (3332.0 ± 137.1 μm^2) was not significantly different from that of the cells washed with PBS alone (3599.0 ± 209.3 μm^2; $P = 0.276$). Thus, removal of bFGF from the cell surface prior to seeding on the coverslips did not affect cell motility. Therefore, under our experimental conditions, single cell migration was not stimulated by contaminating bFGF from previously damaged or dead cells.

To examine whether the bFGF produced by the migrating cells acted via an intracellular mechanism or through release and binding to surface receptor(s), we measured the effects of the addition of exogenous recombinant bFGF (rbFGF) and of neutralizing anti-rbFGF IgG on cell motility. In the first set of experiments phagokinetic track assays with cells of the Zip, B1, and B3 clones were done in the presence of increasing concentrations of rbFGF, from 0.001 ng/ml to 10 ng/ml. As shown in Fig. 3, rbFGF increased the motility of Zip and B1 cells in a dose-dependent manner. Migration of B3 cells, on the contrary, was not significantly affected. The effect of rbFGF on Zip and B1 cells leveled off at 1 ng/ml. At this concentration the level of migration of both clones was equivalent to that of B3 cells. The curve of cumulative frequencies of Zip cells in the presence of 0.1 ng of bFGF per ml was superimposable with that of B1 cells in control medium; the curves of cumulative frequencies of Zip and B1 cells in the presence of 1 ng of bFGF per ml were superimposable with that of B3 cells in the absence of the growth factor (data not shown). This demonstrated that the motility of these cells could be modulated by the extracellular bFGF concentration.

In the second set of experiments we characterized the effect of neutralizing, affinity-purified anti-rbFGF IgG on the motility of B1 and B3 cells. To estimate the amount of purified IgG required to neutralize the bFGF activity, we measured the plasminogen activator-inducing activity of 10 ng of rbFGF per ml on microvascular endothelial cells in the presence of increasing amounts of the antibody. As shown in Fig. 4, 10 μg of purified antibody per ml neutralized the plasminogen activator-inducing activity of 10 ng of rbFGF per ml. On the contrary, the IgG not retained by the rbFGF-Affi-Gel affinity column (flow-through) was ineffective. Single cell phagokinetic assays were done using B1 and B3 cells in the presence of 10 μg of affinity-purified anti-rbFGF IgG or the flow-through IgG per ml or an equivalent volume of PBS. As shown in Fig. 5, in the presence of the irrelevant IgG the mean track area of B1 cells (Fig. 5 *Upper*) was 3780.6 ± 239.0 μm^2 and was not significantly different from the mean track area measured in the absence of IgG (3773.2 ± 212.9 μm^2). The anti-bFGF antibody significantly reduced the mean track area to 1021.5 ± 86.6 μm^2 ($P < 0.001$), a value almost equivalent to that of the Zip cells (887.6 ± 50.5 μm^2). The mean track area of B3 cells (Fig. 5 *Lower*) in the absence of IgG was 7285.7 ± 362.0 μm^2 and was equivalent to that determined in the presence of the flow-through IgG. Addition of the anti-rbFGF IgG significantly reduced the mean track area to 3551.2 ± 269.4 μm^2 ($P < 0.001$). On the contrary, addition of anti-rbFGF IgG did not affect the level of migration of the control Zip cells (Fig. 5 *Lower*). Thus, the motility of B1 and B3 cells was dramatically reduced by specific neutralizing antibody to rbFGF. These results demonstrated that the bFGF produced by viable, migrating cells must be exported into the extracellular environment to stimulate

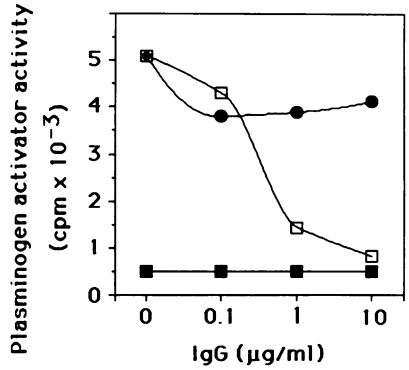

FIG. 4. Neutralization of bFGF-induced plasminogen activator activity of BCE cells by affinity-purified antibody to rbFGF. Affinity-purified anti-rbFGF and irrelevant IgG were tested for their capacity to neutralize the plasminogen activator-inducing activity of 10 ng of rbFGF per ml on BCE cells. The ordinate shows ^{125}I cpm released from a ^{125}I-labeled fibrin layer by BCE cell extracts in the presence of plasminogen. □, Anti-bFGF IgG; ●, flow-through IgG; ■, BCE cells in the absence of bFGF.

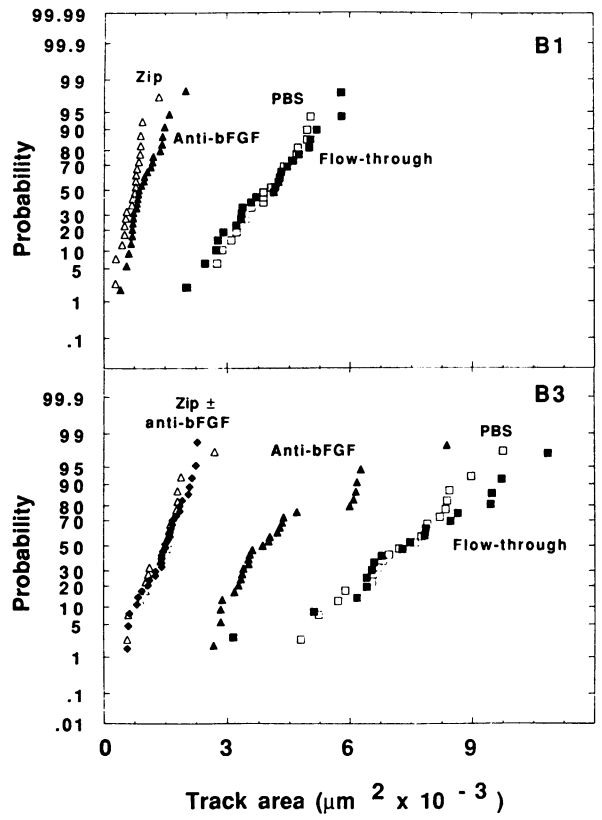

FIG. 5. Effect of affinity-purified anti-bFGF antibody on the motility of B1, B3, and Zip cells. B1, B3, and Zip cells were seeded onto gold-coated coverslips at the concentration of one cell per coverslip. Two hours later the medium was replaced with medium containing either 10 μg of affinity-purified anti-rbFGF IgG per ml or 10 μg of the flow-through IgG per ml from the rbFGF-Affi-Gel chromatography or an equivalent volume of PBS. Following 16 hr of incubation at 37°C, measurement of the phagokinetic track areas and elaboration of the results were performed. (*Upper*) □, B1 cells plus PBS; ■, B1 cells plus flow-through IgG; ▲, B1 cells plus anti-bFGF IgG; △, Zip cells. (*Lower*) □, B3 cells plus PBS; ■, B3 cells plus flow-through IgG; ▲, B3 cells plus anti-bFGF IgG; △, Zip cells; ◆, Zip cells plus anti-bFGF IgG.

migration through interaction with its surface receptor(s) in a true autocrine manner.

DISCUSSION

A unique feature of bFGF is that all forms of its primary translational products lack a hydrophobic signal sequence (29, 30) that would direct their secretion through the "classical" secretory pathway. However, although relatively large amounts of bFGF are found intracellularly (3, 32, 33), several findings indicate that this growth factor acts extracellularly: (*i*) the presence of significant amounts of bFGF in the extracellular matrix *in vitro* and *in vivo* (14, 15, 17, 19, 20), (*ii*) the existence of plasma membrane receptors (15, 21–25), and (*iii*) the observation that neutralizing antibodies to bFGF alter several properties of bFGF-producing cells (26, 27). Thus, the mechanism of bFGF release is a major question in bFGF physiology. Monitoring bFGF release in cell cultures is difficult because the amount of bFGF found in culture fluids is very low and contamination by bFGF derived from damaged or dead cells cannot be excluded. Therefore, it has been impossible to understand the pathway of release and to demonstrate that bFGF is a true autocrine factor—i.e., that it stimulates the cell that produces it.

To address this problem we have employed the phagokinetic track assay (39) to study the effect of neutralizing anti-bFGF antibody on the migration of single, isolated cells. This assay affords accurate, quantitative measurement of cell migration under conditions in which factors derived from cells other than the one being observed can be excluded for the reasons discussed below. In dark-field illumination only actively migrating, and therefore viable, cells produce tracks on the layer of colloidal gold. These cells can be visualized by the gold particles they become covered with or phagocytize during locomotion (39). Dead or damaged cells are not visible. It could be argued that the single cells detected might be contaminated with dead or damaged cells that are not motile and, therefore, not visible. However, the correspondence of the number of single cells observed with the number expected on the basis of a Poisson distribution with a mean of one cell per coverslip makes the probability that this occurs statistically unlikely. Furthermore, even if a motile cell were contaminated by several dead (nonmotile) cells on the same coverslip, the amount of bFGF released from these cells would likely be insufficient to achieve an effective concentration in the medium (see Fig. 3). bFGF contamination might derive from soluble factor present before the cell cultures are trypsinized for the phagokinetic assay. Although dilution of the cell suspension to one cell per ml (100,000- to 1,000,000-fold) should result in negligible concentrations of soluble cell-derived factors, bFGF might remain bound to the cells through interaction with specific receptor(s) and/or to cell surface-associated glycosaminoglycans. However, washing the cells with suramin, which removes bFGF from the cell surface (28), did not alter cell motility. Thus, cell motility appears to be affected only by factors produced by the single cell present in a culture well.

The single-cell phagokinetic assay of the bFGF transfectant clones of NIH 3T3 cells showed a positive correlation of cell motility with the amount of endogenous bFGF. Exogenous rbFGF had a dose-dependent stimulatory effect on the control Zip cells, which produce undetectable amounts of bFGF, and on B1 cells, which produce at least 10-fold less bFGF than B3 cells. In the presence of 1 ng of rbFGF per ml control Zip cells and B1 cells had the same migratory activity as B3 cells. This showed that the differences in cell motility among these clones was dependent upon the level of bFGF present in the extracellular environment. The lack of effect of exogenous bFGF on migration of B3 cells can be explained by two hypotheses that are not mutually exclusive: (*i*) all bFGF receptors may be saturated by the high amount of bFGF produced by these cells (25), and therefore the cells are insensitive to exogenous bFGF, and (*ii*) the migratory activity of B3 cells is maximal and cannot be increased.

Motility of B1 and B3 cells was dramatically reduced by the neutralizing antibody to bFGF but was unaffected by irrelevant IgG. This antibody appears to be specific based upon the following observations: (*i*) it was raised against and affinity-purified with pure rbFGF, (*ii*) in Western blotting of B1 and B3 cell extracts the antibody stained only bands corresponding to bFGF (N. Quarto, personal communication), and (*iii*) the antibody did not affect migration of control cells that produce undetectable amounts of bFGF. Thus, the neutralizing activity of the anti-bFGF antibody was due to its interaction with bFGF. It should be noted that although the antibody reduced the level of migration of B1 cells to that of the control Zip cells, the motility of B3 cells in the presence of anti-rbFGF IgG was reduced considerably, but to a level significantly higher than that of the Zip cells. This discrepancy may be due to several reasons: (*i*) the antibody concentration was not sufficient to completely neutralize all of the bFGF released by B3 cells; (*ii*) the cells were stimulated by soluble bFGF present in mass culture and subsequently removed by washing the cells: this stimulation lasted several hours and could not be inhibited by anti-rbFGF IgG because bFGF was no longer present; (*iii*) a non-bFGF-dependent

mechanism may partly contribute to modulating B3 cell motility; and (*iv*) in these high producer cells bFGF might act also intracellularly. The anti-rbFGF IgG had no effect on the motility of Zip cells. These cells produce extremely low amounts of bFGF (25, 37), which are likely insufficient to stimulate migration (see Fig. 3). Therefore, the low level of migration of the Zip cells probably represents baseline motility independent of bFGF.

To rule out the possibility that bFGF release was artifactually stimulated by manipulation of the cultures we tried different methods of addition of the antibody or irrelevant IgG to the phagokinetic assay. Changing the medium and adding the antibody in a small volume gave similar results (data not shown). Thus, no artifactual release of bFGF occurred due to physical manipulation of the cultures. We cannot rule out that IgG bound to the gold particles was phagocytozed. However, if this happened, the antibody would be segregated in cytoplasmic vesicles and be inaccessible to the cytosolic bFGF.

"Leakage" of bFGF from migrating endothelial cells has been described (35). However, this appears to occur only in a small percentage of cells in the culture. Gold particles are nontoxic for long-term culture of sparse 3T3 cells (39). Therefore, under our experimental conditions the proportion of "leaky" cells was probably not significant. However, we cannot rule out the possibility that release of bFGF only occurs in actively migrating cells or in cells expressing relatively large amounts of bFGF and is not a phenomenon shared by all cells. Our data provide evidence that the bFGF produced by a viable cell is exported into the extracellular environment and stimulates migration of the same cell through interaction with surface receptor(s). This finding raises an interesting question as to the fate of bFGF after it is released from the cell. In our single-cell phagokinetic assay one cell was allowed to migrate in 1 ml of culture medium for 16 hr. Thus, the ratio of the cell volume to the volume of medium was extremely small ($\approx 5 \times 10^{-10}$, assuming a spherical cell with a radius of 5 μm). Under these conditions it appears extremely unlikely that the concentration of bFGF in the culture medium could be in the range of its K_d for the cell receptor(s). Therefore, once released, bFGF must be concentrated on or very close to the cell surface. This can be achieved either through binding directly to the plasma membrane receptor(s) and/or through interaction with cell-bound glycosaminoglycans or other cell surface molecules. Whatever the interaction with the cell surface, bFGF remains accessible to neutralizing antibody. This could explain why migration of B1 cells was not affected by addition of rbFGF concentrations lower than 0.1 ng/ml, whereas motility of Zip cells was stimulated.

Though the intimate molecular mechanism by which bFGF is exported out of the cell remains unexplained, on the basis of our data it can be concluded that bFGF is released by viable cells and is not derived only from cell damage or lysis. The bFGF exported into the extracellular environment is concentrated on the cell surface and stimulates migration of the same cell that secretes it. bFGF therefore acts through a true autocrine mechanism.

This work is dedicated to the memory of our colleague and friend, Dr. Elizabeth Suenson. We thank Dr. N. Quarto for kindly providing the bFGF-transfectant clones, Drs. C. Basilico, G. Kreibich, I. Ivanovic, F. Maxfield, D. Moscatelli, M. Presta, D. Sabatini, and L. Wilson for helpful comments and suggestions, and M. Vassallo and E. Toufexis for skillful technical assistance. This work was supported by grants from the National Institutes of Health and the American Cancer Society to D.B.R. and from the Italian Association for Cancer Research and the Italian Ministry of Scientific Research and University to P.M. The work was carried out during P.M.'s tenure at New York University Medical Center.

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