Alternative patterns of mitogenesis and cell scattering induced by acidic FGF as a function of cell density in a rat bladder carcinoma cell line

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The dual function exerted by acidic fibroblast growth factor (aFGF) in a rat bladder carcinoma cell line has been explored under two different conditions of culture density. At low cell density, aFGF promotes the epithelium-to-mesenchyme transition of NBT-II cells characterized by cell dissociation, morphological changes toward a fibroblastic-like phenotype, and acquisition of cell motility. Under these conditions, NBT-II cells are unresponsive to the growth-promoting effect of aFGF. At high cell density, aFGF is a potent mitogenic factor, but its scattering activity is essentially abrogated. Slight modifications in the binding of aFGF to its specific receptors were observed at high cell density; these changes correlated with a downregulation of receptors with no apparent change in their molecular form. NBT-II cells located at the edge of artificial wounds mimicked the behavior of subconfluent cells, because they did not proliferate upon aFGF treatment. Furthermore, in large-sized NBT-II colonies, peripheral cells were the first to dissociate in response to aFGF. Altogether, our results suggest that the cellular response to multifunctional growth factors might depend on the localization within the responding cell population.

Introduction

Cell migrations take place during various biological processes, including tissue formation and remodeling, wound repair, and inflammation. They are also involved in tumor cell dissemination, which is an integral component of the metastatic process. One of the most puzzling

examples of cell motility is provided by the epithelial-mesenchymal transition (EMT)¹, which has been described in many morphogenetic processes (reviewed in Thiery *et al.*, 1985; Kogalega, 1986; Duband *et al.*, 1987). During EMT, the transient acquisition of locomotory properties is accompanied by dramatic changes in the state of cell differentiation: cells lose their epithelial features and acquire a morphology typical of fibroblastic cells.

Although little is known about the biochemical pathways and effector molecules involved in the process of cell locomotion, it seems likely that different chemical factors influence the migration of cells. In this respect, it has been reported that growth factors, which regulate cell proliferation, can also act as inducer molecules of *in vitro* cell motility: epidermal growth factor (EGF) stimulates the nondirected movement of keratinocytes (Barrandon and Green, 1987), whereas platelet-derived growth factor and transforming growth factor β (TGF β) are both chemotactic for fibroblasts and other cell types (Grotendorst *et al.*, 1981; Grotendorst, 1984; Seppä *et al.*, 1982; Postlethwaite *et al.*, 1987; Wahl *et al.*, 1987). In addition, peptide growth factors might control embryonic EMT: for example, the formation of the heart valves and septa—which is induced by an epithelial-mesenchymal cell transformation of endothelial cells—involves, in part, a member of the TGF β family (Potts and Runyan, 1989). Similarly, the TGF β -related protein, the Müllerian inhibiting substance, is produced by the developing testis and is responsible for the anatomical regression of the Müllerian tract (Cate *et al.*, 1986), a process that is accompanied by an EMT.

It is well documented that growth factors are multifunctional, stimulating a large range of

¹ Abbreviations: aFGF, acidic FGF; bFGF, basic FGF; DMEM, Dulbecco's modified Eagle's medium; DP, desmoplakin; EGF, epidermal growth factor; EGTA, ethylene glycol-bis (β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EMT, epithelial-mesenchymal transition; FCS, fetal calf serum; FGF, fibroblast growth factor; PBS, phosphate-buffered saline; PKC, protein kinase C; TGF β , transforming growth factor β .

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biological activities (reviewed by Sporn and Roberts, 1988). They can induce diverse functions, including cell proliferation and cell motility, which, in certain cases, can be mutually exclusive (e.g., O'Neill *et al.*, 1985). It would thus be of interest to understand how, after interacting with a given growth factor, a cell is directed to enter cell division or to undergo EMT. One attractive hypothesis could be that the dual functions of growth factors result from the presence or absence of cellular effector molecules specifically determining the choice between the two pathways.

We have used the rat bladder carcinoma-derived cell line NBT-II (Toyoshima *et al.*, 1971) as an *in vitro* model system of the reversible conversion of cells from an epithelial to a fibroblastic-like phenotype (Boyer *et al.*, 1989). We have demonstrated that, on addition of acidic fibroblast growth factor (aFGF), epithelial clusters of NBT-II cells dissociate and give rise to individual, elongated, actively migrating cells (Vallés *et al.*, 1990). With an interest in understanding how a growth factor can serve as a mitogen in one context and promote cell motility in another, we have investigated the response of NBT-II cells to aFGF as a function of the environmental conditions of culture. We have established that aFGF has a scattering activity and no growth-promoting effect when added to subconfluent cultures, whereas it induces cell division but no cell dissociation on confluent cultures. This model system offers a unique opportunity to study the reciprocal interactions between growth factors and their cellular targets.

Results

Confluent NBT-II cell monolayers do not undergo EMT upon aFGF treatment

As previously described (Boyer *et al.*, 1989; Vallés *et al.*, 1990), sparse cultures of NBT-II cells assume an epithelial organization in standard conditions of culture. On addition of aFGF, the epithelial clusters readily dissociate, giving rise to individual, fibroblastic-like cells that migrate actively. The overall changes in NBT-II cell morphology, appearing after addition of aFGF, are highly reminiscent of an EMT, i.e., an eventually reversible process of conversion between the epithelial and mesenchymal states. Thus, in our model system, we have used the term EMT to indicate that NBT-II cells lose their epithelial characteristics and acquire some of the properties typical of fibroblastic cells.

Cell scattering induced by aFGF was estimated by the modulation of intercellular junctions, and specifically, by the alteration of des-

mosomes. We had previously shown that the immunoreactive pattern observed with the monoclonal antibody to desmoplakin (anti-DP), which recognizes a subset of proteins of the desmosomal plaque (for review, see Garrod *et al.*, 1990), disappeared from the cell boundaries (Boyer *et al.*, 1989; Vallés *et al.*, 1990). DP immunoreactivity was redistributed as intracellular dots, suggesting that desmosomes were split and internalized during the EMT induced by aFGF in NBT-II cells (e.g., Figure 1D). Cell dissociation began 5 h after addition of aFGF and was achieved in 12 h. It was accompanied by the acquisition of cell motility, as inferred from time-lapse videomicroscopy recordings: epithelial NBT-II cells in standard medium did not move at all, whereas NBT-II cells cultured in the presence of 20 ng/ml aFGF for at least 12 h reached an average speed of locomotion of 30 $\mu\text{m}/\text{h}$ (Vallés *et al.*, 1990). In contrast, when confluent cultures of NBT-II cells were incubated with a range of aFGF concentrations (1–100 ng/ml) for 24 h, no morphological change except cell spreading was observed. DP immunoreactivity remained localized at regions of intercellular contacts, indicating that cells did not dissociate (Figure 1A).

The absence of a scattering effect induced by aFGF at confluency possibly could result from the exclusive localization of receptors specific for aFGF on the basolateral cell membranes that could not be reached from the apical side. NBT-II cells were therefore grown to confluency on polycarbonate filters, thus allowing free access to both basolateral and apical surfaces. Under such conditions, epithelial NBT-II cells formed a dense monolayer of tightly packed cells (Figure 1B); and, after a 2-d culture in presence of 20 ng/ml aFGF added into either the upper or lower chamber delimited by filters, the distribution of DP immunoreactivity was not altered (Figure 1C). Conversely, addition of 20 ng/ml aFGF into either the upper or lower chamber of subconfluent cultures induced a modulation of DP immunoreactivity similar to that observed for sparse cultures on glass (Figure 1, compare D and E).

Absence of a scattering effect by aFGF does not result from lack of space in dense monolayers

We next tested whether the incompetence of aFGF to induce EMT at confluency resulted from the lack of space between cells preventing free cell movement and, consequently, cell scattering. A previous observation did not support this hypothesis: if cell motility was blocked by cul-

Figure 1. Absence of a scattering effect induced by aFGF on confluent NBT-II cell cultures. Cells were cultured on glass (A and D) or polycarbonate filters (B, C, and E) with (A, C, D, and E) or without (B) aFGF. Confluent (A–C) or sparse (D and E) cell cultures were fixed and processed for immunofluorescence microscopy with antibody against DP. Note that addition of aFGF results in cell spreading without any observable effect on DP immunolocalization. Bar, 10 μ m.

turing cells with cytochalasin B, dissociation still occurred (Boyer *et al.*, 1989). Furthermore, in a dense monolayer of fibroblastic-like NBT-II cells grown to confluency in the presence of aFGF, cells were still able to move, exchanging neighbors with one another (speed of locomotion: 25–30 μ m/hr).

We further examined this hypothesis by carrying out another set of experiments. For this purpose, we took advantage of the fact that, whereas cell dissociation monitored by the internalization of desmosomal proteins begins after a lag period of ~ 5 h following addition of aFGF, it requires only a short pulse (3 h) of aFGF for it to be completed 15 h later (Boyer *et al.*, 1989). Moreover, it was also shown that NBT-II cells could retain the inductive signal provided by a 3-h pulse of aFGF after trypsinization and replating: if cells were incubated for 3 h with aFGF, trypsinized, and incubated for 15 h more in the absence of aFGF, DP immunoreactive dots were internalized. In contrast, control cells trypsinized and then cultured with standard medium still expressed DP immunoreactivity at the cell periphery.

NBT-II cells were thus grown to either subconfluency or confluency. aFGF (20 ng/ml) was added for 3 h. Cells were then trypsinized, washed extensively, and replated at low (Figure 2, A, C, and E) or high densities (Figure 2, B and D) and then cultured for another 15 h in standard medium lacking aFGF. At the end of this period, cells were fixed and processed for immunofluorescence microscopy with anti-DP. As control, subconfluent and confluent cultures received a 3-h pulse of aFGF and were immediately processed for immunofluorescence microscopy. In these cultures no desmosome loss was observed (not shown). As already mentioned, confluent and subconfluent cells trypsinized and then cultured in standard medium were able to reform desmosomes at the end of the incubation period (Figure 2, A and B, respectively). Instead, when subconfluent cells were pulsed with aFGF for 3 h, trypsinized, and replated at low (Figure 2C) or high (Figure 2D) densities, cell contacts did not reform, as visualized by the absence of desmosome staining. This result suggested that these cells transplanted into a confluent state were able to dissociate, pro-

Figure 2. The absence of desmosome internalization after addition of aFGF in confluent cultures cannot be accounted for by the lack of space available for cell migration. NBT-II cells were grown to subconfluency (A, C, and D) or confluency (B and E) in standard medium (A and B) or in the presence of aFGF (20 ng/ml) added during the last 3 h of culture (C–E). Cells were then trypsinized and replated at low (A, C, and E) or high (B and D) densities for 15 h in standard medium. Cell cultures were fixed and processed for immunofluorescence microscopy using an antibody against DP. Bar, 10 μ m.

vided that the scattering signal had already been initiated by aFGF in the previous subconfluent state. Conversely, if confluent cells were pulsed with aFGF, trypsinized, and cultured as sparse colonies, a high proportion of them remained interconnected by desmosomes, as revealed by the immunoreactive pattern (Figure 2E). However, desmosomes were modulated in some cells that had probably been able to initiate the dissociating signal provided by aFGF at confluency.

aFGF is not mitogenic for subconfluent NBT-II cells

In a previous study, we had shown that aFGF is a potent promoter of proliferation of confluent

NBT-II cells (Vallés *et al.*, 1990). Because the scattering effect of aFGF was observed for subconfluent cultures only, we have sought to determine whether the mitogenic function of aFGF on NBT-II cells could depend on the degree of confluency.

In a first set of experiments, we measured [3 H]thymidine incorporation into NBT-II cells at low and high cell densities (Figure 3). At confluency (Figure 3A), NBT-II cells were better stimulated to enter DNA synthesis by aFGF than by 10% fetal calf serum (FCS). The stimulus provided by aFGF was dose-dependent, as revealed by the differences in [3 H]thymidine incorporation of NBT-II cells stimulated by 10 or 1 ng/ml aFGF (see also Vallés *et al.*, 1990). In

A **B**

Figure 3. [^3H]thymidine incorporation of sparse and confluent NBT-II cell cultures. Cells were seeded in 24-well plates in standard medium at high (A) or low (B) densities so that they reached confluency (A) or were only 30% confluent (B) after 2 d culture. They were serum-starved (0.5% FCS) for 24 h (A) or 18 h (B). Different concentrations of growth factors or 10% FCS were then added for 24 h (A) or 18 h (B). Unless indicated, aFGF was used at 10 ng/ml and bFGF at 30 ng/ml. The figure shows one typical experiment. The [^3H]thymidine incorporation was determined independently in A and B and was expressed as the percentage of the average maximum incorporation. The maximum value of counts per minute incorporated was $222\,721 \pm 22\,354$ in A and $330\,310 \pm 46\,850$ in B. Each column represents the mean value of three identical wells $\pm$ SD.

addition, when fresh medium containing 10% FCS was mixed with aFGF, the effects observed with the two growth promoting agents were potentiated. As already reported (Vallés *et al.*, 1990), basic fibroblast growth factor (bFGF),

which does not induce the EMT of subconfluent NBT-II cells, was also a growth-stimulating factor. The fact that bFGF does not have an effect on EMT but stimulates mitogenesis may reflect functional differences mediated by different receptor species or signaling pathways.

In contrast, subconfluent NBT-II cells did not incorporate [^3H]thymidine after incubation with aFGF (Figure 3B). Compared with the basal level of [^3H]thymidine incorporation, the addition of aFGF to sparse cultures even reduced the amount of counts per minute incorporated. A large range of concentrations of aFGF (0.01–100 ng/ml) was tested without any observable stimulating effect on DNA synthesis of subconfluent NBT-II cells (not shown). In initial experiments, the total incubation time in low serum (0.5%) was 48 h for sparse and confluent cultures. However, numerous dead cells detaching from the substrate were observed in sparse cultures. This prompted us to modify the experimental protocol. Reducing the total incubation time to 36 h did not result in an increase in [^3H]thymidine incorporation of subconfluent cultures induced by aFGF. Under these experimental conditions, the number of cells detached from the substrate at the end of the experiment was low and cell viability was excellent. Thus neither cell detachment nor cell death was responsible for the lack of stimulation by aFGF on sparse cultures. Moreover, subconfluent NBT-II cells were able to incorporate [^3H]thymidine in response to stimulation by 10% FCS, ruling out the possibility that sparse NBT-II cells partially deprived of serum for 18 h would be intrinsically unable to divide. It is noteworthy that addition of 10% FCS had a more pronounced stimulating effect on sparse than on confluent NBT-II cells (Figure 3, compare columns 2 of A and B). The stimulation by a mixture of aFGF and 10% FCS did not have the synergistic effect on DNA synthesis that was observed for confluent cultures. Instead, addition of aFGF had a significantly inhibitory effect on the [^3H]thymidine incorporation of sparse NBT-II cells stimulated by FCS.

Incubation of sparse cultures with the conditioned medium of confluent cultures in conjunction with aFGF did not promote [^3H]thymidine incorporation. bFGF was also unable to stimulate [^3H]thymidine incorporation; and it did not have the inhibitory effect of aFGF tested in the same conditions, as shown by the level of incorporation in the presence of bFGF alone or of bFGF plus FCS. aFGF and bFGF, used in combination at concentrations that were mitogenic for confluent cultures, were also ineffective.

Figure 4. Specific binding of ^{125}I -aFGF to NBT-II cells as a function of cell density. (A) Sparse (3.2×10^5 cells/cm 2) and confluent (2×10^6 cells/cm 2) cultures were incubated for 4 h at 4°C with the indicated ^{125}I -aFGF concentrations alone or in the presence of 1000-fold molar excess of unlabeled aFGF. Specific cell-associated binding of sparse (□) and confluent (◆) cultures is represented. (B) Scatchard analysis of ^{125}I -aFGF binding to sparse and confluent NBT-II cells. The amounts of bound and free ^{125}I -aFGF were plotted according to the method of Scatchard (1949). Each point represents the mean of duplicates of a representative experiment which varied less than 10%. Sparse (□) and confluent (◆) conditions.

Altogether, these results demonstrated that subconfluent NBT-II cells were unable to enter DNA synthesis in response to aFGF or bFGF stimulation, although they were readily able to enter mitosis after stimulation by serum. Interestingly, aFGF was able to potentiate the response of confluent cells stimulated by FCS, whereas it inhibited the FCS-induced mitogenesis at subconfluency.

A second way to assess the absence of a mitogenic activity of aFGF on sparse cultures was to test cell proliferation. Addition of either aFGF or bFGF to cultures grown in 2.5% FCS was not found to increase cell division of NBT-II cells, whereas under these conditions, aFGF was the only growth factor to have a dissociating effect. A range of concentrations tested (0.1, 1, 10, 20, 30, 50, and 100 ng/ml) gave similar results (data not shown). After several days in culture, two

opposite effects were always observed: the addition of bFGF in medium containing 10% FCS promoted a slight increase in the number of cells, whereas aFGF in medium containing either 2.5% or 10% FCS inhibited cell proliferation.

Effects of cell density on aFGF binding to NBT-II cells

The observation that aFGF elicits dual, density-dependent responses in NBT-II cells prompted us to examine in more detail the receptors for aFGF under conditions favoring either cell dissociation or mitogenesis. We first determined the amount of cell surface-bound aFGF as a function of cell density and of aFGF concentration. As shown in Figure 4A, at saturating concentrations, sparse NBT-II cells bound twice as much ^{125}I -aFGF/cell than cells at confluent densities. A similar decrease in ^{125}I -aFGF binding to confluent cells was observed in cultures grown in the presence of 10 ng/ml of aFGF (data not shown). Scatchard analysis of equilibrium binding data revealed that confluent NBT-II cells expressed $\sim 13\,000 \pm 1400$ receptors/cell with a dissociation constant (K_d) of 34 ± 8.5 pM. Likewise, sparse cells expressed $\sim 14\,000 \pm 1500$ receptors/cell with a K_d of 14 ± 4 pM (Figure 4B). Thus, only a slight decrease in the dissociation constants of high-affinity receptors present in sparse and confluent cells could be observed. Similarly, a large number of low-affinity binding sites, $\sim 3.7 \times 10^6$, were also detected, with binding constants of 188 ± 56 and 390 ± 170 nM for sparse and confluent cultures, respectively (Figure 4B). However, in our system, the biological significance of these low-affinity sites is not clear.

We sought to determine whether the decrease in ^{125}I -aFGF binding on confluent cultures was associated with a lack of access to basolateral receptors. Sparse and confluent cultures were incubated before ^{125}I -aFGF binding in calcium-free medium containing 1 mM ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA) to disrupt intercellular contacts. Cells in monolayers incubated for 30 min in this medium rounded and lost contacts with their neighbors. As shown in Figure 5, no difference in the amount of ^{125}I -aFGF binding was detected in EGTA-treated and nontreated confluent cultures. Sparse cultures bound relatively more factor in EGTA-containing medium than in standard medium.

Affinity cross-linking reveals no difference in receptor forms in sparse versus confluent cultures

To investigate the possibility that distinct cell surface receptors might be responsible for each

Figure 5. Decrease in ^{125}I -aFGF binding on NBT-II cells is not due to a lack of access to basolateral receptors. Sparse ($3\text{--}5.7 \times 10^5$) and confluent (2×10^6) NBT-II cells were pretreated in calcium-free medium containing EGTA for 30 min before ^{125}I -aFGF (2 ng/ml) binding. Specific cell-surface binding is expressed in relation to cell density. ▨, EGTA-treated cultures; □, nontreated cultures.

of the effects observed, cross-linking experiments were performed. Affinity labeling of aFGF receptors on NBT-II at different densities revealed no difference in the pattern of cross-linked products: confluent and sparse NBT-II cells expressed predominantly receptor-ligand complexes with M_r of 150 and 130 kDa (Figure 6). A third complex with M_r of 180 kDa is also resolved in both conditions (Vallés *et al.*, 1990). Instead, a reduction in the amount of radioactivity associated with these forms was visualized under confluent conditions. The apparently greater diminution (~ 10 -fold) of aFGF binding, compared with the twofold reduction observed in the equilibrium binding studies (see Figure 4A), probably results from the nonsaturating concentration (2 ng/ml) of ^{125}I -aFGF used.

^{125}I -aFGF binds on subconfluent NBT-II cells that do not synthesize DNA after stimulation by aFGF

To visualize the differential binding of aFGF and its opposite growth-promoting effects on subconfluent versus confluent cultures of NBT-II cells, we performed a series of autoradiographs in various conditions of culture. As shown in Figure 7, A and B, after incubation with 5 ng/ml ^{125}I -aFGF, radiolabeled material heavily stained the surface of subconfluent cells but was almost undetectable on confluent cultures. ^{125}I -aFGF was almost totally displaced by an excess (1000 $\times$) of native aFGF indicative of the binding specificity (not shown).

In contrast, few subconfluent cells incorporated ^3H thymidine—visualized as dark, dense

material within the nuclei—in response to incubation with 20 ng/ml aFGF, whereas numerous cells were heavily stained in confluent cultures stimulated by aFGF under the same experimental conditions (Figure 7, C and D). As a control, nuclei of confluent or subconfluent cells incubated with 10% FCS were radiolabeled by ^3H thymidine (Figure 7, E and F).

Autoradiography of ^3H thymidine-labeled wounded cultures

Because autoradiography of cells with ^3H -thymidine incorporated into their nuclei is a good assay to determine what populations of cells respond to aFGF by DNA synthesis, we performed this test on in vitro wounds of NBT-II cell monolayers. In an artificially produced wound, there are two populations of cells, characterized by their relationships with the neigh-

Figure 6. Affinity labeling of aFGF receptor to NBT-II cells as a function of cell density. Sparse (lane a) and confluent (lane b) cultures were incubated with 2 ng/ml of ^{125}I -aFGF in the absence (lanes a and b) or in the presence (lane d, sparse cultures) of 100-fold molar excess of unlabeled aFGF using disuccinimidyl suberate (DSS) as the cross-linking agent. Lane c, without DSS. Receptor-ligand complexes were resolved on 5–10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualized by autoradiography.

Figure 7. Differential binding of ^{125}I -aFGF and $[^3\text{H}]$ thymidine incorporation visualized in sparse and confluent cell cultures. Confluent (A, C, and E) and sparse (B, D, and F) NBT-II cell cultures were established as described in Methods. ^{125}I -aFGF (5 ng/ml) was added for 3 h at 4°C (A and B) and autoradiography was performed after extensive washes and fixation of cell cultures. Before $[^3\text{H}]$ thymidine incorporation (C–F), cells were deprived of FCS, by culture in medium containing 0.5% FCS, and then stimulated by aFGF (C and D) or 10% FCS (E and F). Bar, 10 μm .

boring cells: the lateral membranes of cells located at the edge of the wound are only partially engaged in intercellular contacts, whereas cells located in the non-wounded area are entirely surrounded by and interconnected to other cells. We investigated whether the differences between these two subsets of cells, differing by their localization in the wounded culture, might reflect the opposition between confluent and sparse cells. FCS but not aFGF was able to

stimulate DNA synthesis of cells located at the edge of the wound, whereas addition of aFGF to a culture stimulated by FCS resulted in a slight decrease of $[^3\text{H}]$ thymidine incorporation (Figure 8). In apparent contrast to the observations reported previously (Boyer *et al.*, 1989), there was a striking reduction in the migration of individual cells within the wound incubated with aFGF. This reduction resulted most likely from serum starvation—versus no serum de-

Figure 8. $[^3\text{H}]$ thymidine incorporation of wounded cultures of NBT-II cells. Confluent NBT-II cell cultures in standard medium containing 10% FCS were wounded and cultured in medium containing 0.5% FCS for 18 h followed by an 18-h incubation with 20 ng/ml aFGF (A), 10% FCS (B), or aFGF plus 10% FCS (C). $[^3\text{H}]$ thymidine was added in each culture for the last 4 h and then processed for autoradiography. Dark cells represent cells that have entered DNA synthesis. Bar, 100 μm .

privation in the previous report—thus providing suboptimal conditions for cell locomotion.

Dissociation of NBT-II colonies depends on their size

We further studied whether the scattering response of peripheral and inner cells of NBT-II clusters induced by aFGF is different and might reproduce the differential behavior of confluent and subconfluent cells. NBT-II cells were thus cloned to avoid cell heterogeneity within a cluster. This procedure allowed us to make a correlation between the extent of cell dissociation and the size of the cluster. After 4–7 d in culture, colonies of different sizes were selected and stimulated by aFGF for 5 h before processing for immunofluorescence microscopy with anti-DP. This time was chosen because it permitted us to visualize the cells leaving the cluster. Small colonies incubated in medium containing aFGF were entirely dissociated at the end of the incubation time, as demonstrated by the absence of DP immunoreactivity at the cell periphery (Figure 9B). Control colonies in standard medium did not exhibit DP internalization (Figure 9A). Larger colonies were not completely dissociated after 5 h of incubation (Figure 9C). Interestingly, cells with modulated DP immunoreactivity pattern were peripheral cells, whereas inner cells retained their membrane-bound DP immunoreactivity. The number of inner cells that remained cohesive was dependent on the colony size (Figure 9, compare C and D). As expected, 18 h of culture in the presence of aFGF entirely dissociated larger colonies (data not shown).

Discussion

Our results demonstrate that aFGF induces alternative effects on NBT-II cells depending on the growth status of the cell: on subconfluent cultures, aFGF promoted EMT and growth arrest, whereas on confluent cultures, it only stimulated DNA synthesis. We have ruled out the possibility that the absence of the mitogenic effect of aFGF on subconfluent cells could be due to the intrinsic inability of NBT-II cells to reenter mitosis, because FCS is a potent growth-promoting agent under the same experimental conditions. Moreover, absence of a scattering effect at confluency is not a consequence of crowding and thus does not seem to depend on external constraints. Finally, the same range of aFGF concentrations (10–20 ng/ml) is effective on cell proliferation at confluency and EMT at subconfluency, suggesting that the dual effects observed are dependent on cell density and not on concentration of aFGF. Therefore, the cell status appears to dictate the responsiveness of NBT-II cells to aFGF.

It is noteworthy that differences in cell density imply both modifications in cell cycle and in cell architecture. In general, confluent cells are quiescent, whereas subconfluent cells are continuously cycling. On the other hand, the organization of the cytoskeleton depends on the extent of cell contacts generated in culture (Bré *et al.*, 1987; Buendia *et al.*, 1990): sparse epithelial cells are not polarized and are characterized by a high instability of some of their cytoskeletal components. In contrast, epithelial cells grown to confluency become polarized and

Figure 9. Small colonies of NBT-II cells dissociate faster than larger colonies. NBT-II cells were cloned by limiting dilution. Four to 7 d after cloning, colonies of different sizes were selected. aFGF (20 ng/ml) was added for 5 h before fixation and immunofluorescence microscopy with anti-DP (B–D). As a control, DP immunolocalization was studied also in NBT-II colony cultured without aFGF (A). Note the internalization of DP immunoreactivity in cells of a small colony (B), the persistence of DP dots at the cell boundary between two central cells in a colony of intermediate size (arrow in C), and the numerous immunofluorescent intercellular contacts remaining between cells located in the center of a large colony (D). Bar, 10 μ m.

stabilize their cytoskeletal elements. Thus, the opposite effects induced by aFGF on subconfluent versus confluent NBT-II cultures could be accounted for by differences in either growth rate or cell shape as described in other systems (Gerlach *et al.*, 1989). Our results, obtained with the wound-healing assay, favor the interpretation that spatial organization and cell shape of a population play a major role in the cell density-dependent response to aFGF. Density-dependent inhibition of mitogenesis promoted by growth factors has already been reported (Paulsson *et al.*, 1988; Goodman and Majak, 1989). In vascular smooth muscle cells, growth inhibition induced by TGF β has been correlated with distinct TGF β receptor subtypes (Goodman and Majak, 1989). Moreover, it has been noted that cell density could also influence growth factor receptor downregulation (Rizzino *et al.*, 1988; Gerlach *et al.*, 1989). Rizzino and collaborators observed a correlation between cell density and receptor binding, which was related to a decrease in high-affinity-receptor number, whereas postconfluent endothelial cells modulate both tumor necrosis factor receptor number and affinity with an apparent change in the receptor form (Gerlach *et al.*, 1989).

Consistent with recent evidences that growth factors can exert multiple effects, we have observed two different biological activities of aFGF on NBT-II cells, that is, mitogenesis or EMT. The growth factor multifunctionality has been under intensive study with a particular emphasis on the effects on cell differentiation and motility. For example, fibroblast growth factor (FGF) induces lens-fiber differentiation or proliferation in a dose-dependent manner (McAvoy and

Chamberlain, 1989), whereas it inhibits adipocyte and myocyte terminal differentiation (Olwin and Hauschka, 1988; Navre and Ringold, 1989). Similarly, TGF β blocks myoblast fusion without affecting either the proliferative response or the induction of muscle-specific genes (Hu and Olson, 1990). On the other hand, growth factors are known to stimulate motility (O'Neill *et al.*, 1985) and chemotaxis (Seppä *et al.*, 1982; Grotendorst, 1984; Wahl *et al.*, 1987; Orr *et al.*, 1988; Stracke *et al.*, 1988). Likewise, EGF induces extensive morphological changes in human carcinoma cells (Chinkers *et al.*, 1981), which are associated with the redistribution of cytoskeletal elements (Schlessinger and Geiger, 1981). Compared with previous reports, our model system offers a unique opportunity to study the balance between two different biological responses induced by aFGF in opposite situations.

We have explored whether aFGF exerts dual effects on NBT-II cells by interacting with functionally distinct cell-surface receptors by the use of affinity-labeling and ligand-binding assays. It appeared that the absence of a dissociating effect is correlated with slight changes in binding properties of the high-affinity receptor and not with receptor display on the surface of confluent cells. The modification in aFGF binding is related to an apparent decrease in affinity with no reduction in the number of receptors. Also, changes in cell responses seem not to result from the expression of a new receptor form, even though the presence of different receptors of similar apparent molecular weight cannot be ruled out. In conclusion, because only limited modifications of aFGF receptor properties were

observed, a more likely explanation for the alternative biological effects exerted by aFGF on NBT-II cells is the existence of distinct signaling pathways. In this respect, it has been postulated that growth factors could be divided into two classes: one class stimulates a rise in cyclic adenosine monophosphate and inhibits cell motility, whereas the second class of growth factors induces a rise in intracellular Ca^{2+} concentration and promotes cell locomotion (Rozengurt, 1982; McNeil and Taylor, 1987). Furthermore, alternative pathways leading to distinct biological activities have been proposed. The mitogenic activity of bFGF on fetal bovine aortic endothelial cells is dependent on protein kinase C (PKC), whereas bFGF-induced production of plasminogen activator is insensitive to drugs that downregulate PKC activity (Presta *et al.*, 1989). Consistent with these reports, we expect to observe differences in the signaling pathways leading to mitogenesis and cell scattering of NBT-II cells.

Our system reveals opposite effects of aFGF depending on the cell density in culture. To approach the biological reason that a growth factor could elicit differential responses, we have performed wound-healing experiments. They have suggested that NBT-II cells bordering the wound behave like subconfluent cells, i.e., they are growth inhibited in the presence of aFGF. Previous observations demonstrated that NBT-II cells stimulated by an aFGF-containing medium were able to migrate and repair an artificially produced wound (Boyer *et al.*, 1989). In other systems, growth factors have also been implicated in tissue reorganization during the wound-healing process (Buckley *et al.*, 1985; Lynch *et al.*, 1987; Tsuboi and Rifkin, 1990). More strikingly, it has been shown recently that subcutaneous administration of TGF β could substitute completely for wounding, thereby facilitating tumor formation in Rous sarcoma virus-infected animals (Sieweke *et al.*, 1990).

Growth factors have also been implicated in tumor formation and progression. Of interest is the *in vivo* demonstration that the sustained overexpression of the FGF-related gene *int-2* in transgenic mice results in the development of prostatic hyperplasia and mammary hyperplasia that led, in one case, to neoplasia (Muller *et al.*, 1990). Growth factors are also likely to be involved in tumor invasion and metastasis because they can promote tumor cell motility (Orr *et al.*, 1988; Stracke *et al.*, 1988; Tsuboi *et al.*, 1990) and proteinase production (Presta *et al.*, 1988; Tsuboi *et al.*, 1990; Gavrilović *et al.*, 1990). In our *in vitro* model system, aFGF dramatically enhances the invasiveness of NBT-II cells into

three-dimensional collagen gels (G.C. Tucker, unpublished results). Thus, our finding that subconfluent cells mimic the response of peripheral cells in NBT-II colonies suggests that, in the progression of tumors and metastases, the peripheral tumor cells might respond to a given growth factor by escaping from the tumor mass, whereas inner cells, which are not able to scatter, proliferate in response to the same growth factor.

Methods

Reagents

Human recombinant aFGF (Jaye *et al.*, 1987) was kindly provided by Dr. M. Jaye (Rorer Biotechnology, King of Prussia, PA). Bovine recombinant bFGF was purchased from Amersham (Amersham International, Buckinghamshire, UK). Mouse monoclonal antibody against desmosomal proteins DPs I and II was the generous gift of Prof. W.W. Franke (German Cancer Research Center, Heidelberg, FRG). Texas Red-coupled goat anti-mouse IgG (Immunotech, Marseille, France) was used as secondary antibody. EGTA was purchased from Sigma (St. Louis, MO). FCS was from Biological Industries (Kibbutz Beth Haemek, Israel).

Cell culture

The NBT-II cell line, originally established by Toyoshima *et al.* (1971), was obtained from Prof. M. Mareel (University Hospital, Ghent, Belgium). The original tumor from which NBT-II cell line was derived is a squamous bladder carcinoma obtained by chemical carcinogenesis. Under standard conditions of culture, the cells assume an organization and phenotype typical of epithelial cells. The cells were routinely grown (37°C in 5% CO_2) in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated FCS, as described previously (Boyer *et al.*, 1989). Sparse/subconfluent/low-density cultures are defined by 30–50% cell density, compared with 100% cell density for confluent/high-density cultures. When needed, cells were grown on polycarbonate filters (6.5-mm diameter, Transwell, Costar, Cambridge, MA) as described (Bomse *et al.*, 1989). In these experiments, cell density was assessed by cytochemistry using the nuclear dye Hoechst 33258 (Boehringer Mannheim, Mannheim, FRG): filters were fixed at –20°C with methanol for 5 min followed by acetone for 1 min before incubation for 10 min with the dye. Fluorescent nuclei were visualized under epi-illumination with the use of a Leitz Orthoplan microscope.

Cell motility assay

Cells were seeded on glass coverslips and cultured for 2 d in standard medium. Coverslips were mounted in a Sykes-Moore chamber (Bellco Glass, Vineland, NJ) and time-lapse videocinematography was performed over 18 h after adding aFGF at 20 ng/ml in the presence of heparin (10 $\mu\text{g}/\text{ml}$). Migration pathways were plotted on a television monitor screen and traced onto transparent paper. The speed of locomotion was computed as the total track length divided by the duration of the film.

Indirect immunofluorescence microscopy

Cells were seeded on glass coverslips. aFGF (20 ng/ml) plus heparin (10 $\mu\text{g}/\text{ml}$) was added 2 d after plating, and cells

were processed for immunocytochemistry as described previously (Boyer *et al.*, 1989).

Binding studies

Human recombinant aFGF (Jaye *et al.*, 1987) was labeled by a modification of the chloramine-T method (Hunter and Greenwood, 1962) as described (Olwin and Hauschka, 1990). Specific activities of the iodinated ligand varied from 960–2150 dpm/fmol of FGF.

Equilibrium binding assays on NBT-II cells were carried out as described previously (Vallés *et al.*, 1990). In one set of experiments, sparse and confluent cells were preincubated in a calcium-free medium (Hanks balanced salts without Ca^{2+} and Mg^{2+} , GIBCO, Grand Island, NY) containing 1 mM EGTA for 30 min at 37°C before the binding assay. The affinity and number of receptors were determined according to Scatchard (1949) using the LIGAND program (Munson and Rodbard, 1980).

Affinity-labeling assay

Cross-linking of labeled aFGF to sparse (2.6×10^6) and confluent (7×10^6) NBT-II cells grown on 28-cm² dishes was carried out using the method described previously (Vallés *et al.*, 1990). Labeled aFGF was used at 2 ng/ml and competed with a 100-M excess of native aFGF to demonstrate its specificity.

Cell-growth assay

NBT-II cells were seeded at a density of 1×10^5 cells/cm² in 35-mm dish in DMEM medium containing heparin (10 µg/ml) and either 2.5% or 10% FCS. Factors were added on day 1 and then every other day. At days 2, 3, 4, and 5, cells were harvested by gentle trypsinization and counted with a hemocytometer.

Tritiated thymidine incorporation

Liquid scintillation counting. Cells were seeded in 24-well Costar dishes and grown in standard medium for 2 d. The medium was changed to DMEM containing 0.5% heat-inactivated FCS for 24 h (confluent cultures) or 18 h (subconfluent cultures). The cells were then incubated with growth factors or 10% FCS for 24 h (confluent cultures) or 18 h (subconfluent cultures). [methyl-³H]thymidine (specific activity, 42 Ci/mmol, Amersham) was added at 1 µCi/ml for the last 4 h of the experiment. At the end of the labeling period, each culture dish was washed twice with ice-cold phosphate-buffered saline (PBS), followed by the addition of 1 ml ice-cold 10% trichloroacetic acid and incubated for 30 min at 4°C. After four washes with water, cells were solubilized with 0.1 N sodium hydroxide, and radioactivity was quantitated by liquid scintillation counting. Experiments were conducted in triplicate cultures. Results are expressed as the means $\pm$ SD of the radioactivities counted in control and growth factor-treated cultures.

Autoradiography of wounded and nonwounded cultures. NBT-II cell monolayers were obtained after 48 h culture in standard medium on glass coverslips. For "in vitro wound models," confluent monolayers were gently wounded by creating an $\sim$ 1-mm-wide slash through the cell sheet using a Gilson pipette yellow tip and extensively rinsed with medium to remove all cellular debris. That procedure left a cell-free area ("wound"). The following procedure was then identical for wounded and nonwounded cultures.

The medium was changed to DMEM with 0.5% FCS for 18 h. The cells were then incubated with aFGF (20 ng/ml) or 10% FCS as a control for 18 h. Cultures were labeled by the addition of 1 µCi/ml [methyl-³H]thymidine (Amersham)

to the medium during the last 4 h. At the end of the incubation time, cultures were fixed with 3% formaldehyde in PBS. The cultures were coated with a 1/1 dilution of LM emulsion (Amersham). Autoradiographs were exposed for 4 d and developed with Microdol developer (Kodak, Rochester, NY). Preparations were photographed on Ektachrome 50 film (Kodak) with an Olympus camera mounted on a Leitz light microscope.

Binding of radiolabeled aFGF and autoradiography

NBT-II cell cultures on glass coverslips were incubated with DMEM for 3 h at 37°C and for 30 min at 4°C. The cells were then washed three times with binding buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 0.9 mM CaCl_2 , 0.8 mM MgCl_2 , 3 mM KCl, 0.14 M NaCl, 0.1% bovine serum albumin, pH 7.2) and incubated with 5 ng/ml [¹²⁵I]-labeled aFGF. After 3 h incubation at 4°C, cell cultures were washed three times with binding buffer and once with washing buffer (1 M NaCl in binding buffer) for 3 min (Moscatelli, 1987). Cell cultures were fixed with 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.0. Autoradiography was carried out as described for [³H]thymidine incorporation except that cultures were coated with a 1/2 dilution of K-5 emulsion (Ilford, Mobberley, Cheshire, UK). The specificity of the binding was tested in controls in which the binding of 5 ng [¹²⁵I]-labeled aFGF was displaced by an excess (2.5 µg/ml) of unlabeled aFGF.

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