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A minority of carcinoma cells producing acidic fibroblast growth factor induces a community effect for tumor progression

(cell interactions/cooperativity/dissociating factor/metastasis)

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ABSTRACT **It is generally accepted that primary tumors become heterogeneous as a consequence of tumor-cell genetic instability. Clonal dominance has been shown to occur in some experimental models allowing a subpopulation of cells to overgrow the primary heterogeneous tumor and to metastasize. Alternatively, interactions among coexisting tumor subpopulations may contribute to the emergence of a malignant invasive primary solid tumor. We asked the question whether emergence of carcinoma cells producing a growth/dissociating factor within a tumor cell population may be a determinant for tumor progression and for clonal dominance. To mimic such a situation, we have investigated the impact of tumor subpopulation heterogeneity in an *in vivo* model in which mixtures of carcinoma cells that differ in their ability to produce acidic fibroblast growth factor are injected into nude mice. Our data indicate that a growth-factor-producing cell subpopulation can confer increased tumorigenicity to an entire cell population and subsequently elicit a shorter delay for appearance of metastasis. A community effect via cell interactions may account for a heterogeneous tumor cell population rather than clonal dominance during progression of certain tumor types.**

Solid human tumors arise after malignant transformation, giving rise to a heterogeneous and dynamic primary tumor cell population (1, 2) that may progress toward variable degrees of clonality from monoclonal to polyclonal phenotypes (3). It is documented in experimental primary tumors that clonal dominance of one tumor cell subpopulation over others may occur and result in an autonomous overgrowth of that subpopulation (4, 5). However, the mechanisms by which a tumor progresses may be more subtle and can result from cell–cell interactions between variants coexisting in the primary tumor without complete dominance (6). Furthermore, composite tumors may result from balanced or unbalanced growth of several cell subpopulations (7). Interactions between distinct cells of a tumor may take place through mediators such as growth factors, since the role of growth factor signaling pathways has been documented in tumorigenesis (8). However, it has not been shown that the sustained production of a growth factor is a determinant for the maintenance of a cancer phenotype and induction of the metastatic process. To understand the potential role of acidic fibroblast growth factor (aFGF) in the very early event(s) of cancer dissemination, we have set up a model system using the rat bladder carcinoma cell line NBT-II. Upon addition of exogeneous aFGF to sparse cultures *in vitro*, these epithelial NBT-II cells, which do have high-affinity aFGF receptors, scatter and morphologically convert to mesenchymal cells (9, 10), indicating that in this system aFGF acts as a dissociating/scatter factor on epithelial cells. In sharp contrast, aFGF induces mitogenicity at high cell density (11). These cells acquire new morphological characteristics and invasive and metastatic potential when they are rendered autocrine for aFGF (12, 13).

To define the potential role of a few growth-factor-producing cells on tumor progression, we have investigated the impact of tumor subpopulation heterogeneity in an *in vivo* model. Mixtures of carcinoma cells differing in their ability to produce aFGF were injected into nude mice. (*i*) We demonstrate that production of aFGF confers high tumorigenic and subsequently disseminating properties to the producer carcinoma cells. (*ii*) We found that mixtures of aFGF-producing and nonproducing cells behave essentially as cells producing the growth factor in nude mice. (*iii*) We demonstrate that aFGF-producing cells have no selective advantage among the nonproducers either for tumor proliferation or for metastatic spreading, indicating that within a tumor, the cells behave as a dynamic ecosystem with a permanent "cross-talk" between each other as a result of aFGF signaling, conferring tumorigenic and metastatic properties to all the tumor cells by cooperativity.

MATERIALS AND METHODS

Cells. The NBT-II cell line, originally established by Toyoshima *et al.* (14) from a chemically induced rat squamous bladder carcinoma, was obtained from M. Mareel (University of Ghent, Ghent, Belgium). These epithelial cells do not produce endogeneous aFGF but possess a high-affinity aFGF receptor on their surface (10). aFGF-producing NBT-II cells were obtained after transfection with a recombinant plasmid allowing expression of the human growth factor as reported elsewhere (12). The producing clone named NSF14 is no longer epithelial but has a fibroblastic morphology and produces 166 ng of aFGF per 10^7 cells. The growth factor is not secreted because aFGF is devoid of a signal peptide but can be immunoreactively found associated with the extracellular matrix in culture conditions (12).

The NBT-II epithelial cells were tagged with the β-galactosidase reporter gene by transfection with the pCH110 plasmid (Amersham) along with the pAG60 recombinant neomycin-resistance expression vector (15), allowing discrimination of this cell population *in vivo* among other cells.

Tumorigenicity in Nude Mice. Six-week-old female nude mice (*nu/nu* Swiss strain) were subcutaneously injected in the flank with control NBT-II cells, NSF14 aFGF-producing cells, or mixtures of these two cell populations consisting of NBT-II cells with decreasing amounts of NSF14 cells. In all cases, each animal was injected with a constant total number of 3.5×10^6 cells suspended in 0.1 ml of phosphate-buffered saline, unless otherwise specified. Tumor volumes were

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Abbreviation: aFGF, acidic fibroblast growth factor.

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monitored every 2 days or every week and calculated as the volume of a spheroid with a radius corresponding to the mean of the two perpendicular dimensions. After euthanasia, tumors were aseptically removed; animals were routinely examined for metastatic macroscopic lesions in various internal organs.

Detection of Micrometastases. Micrometastases correspond to rat carcinoma cells that have disseminated from the primary tumor but that cannot yet be detected by conventional anatomopathological microscopic observation. Micrometastases were detected after *in vitro* culture of the lymph nodes or other organs (spleen, heart, bladder, liver, or lung). Briefly, a fragment of the organ or the axillary, mesenteric, and inguinal lymph nodes was excised, cut into small pieces, trypsinized, and grown *in vitro* in standard culture conditions used for NBT-II and derivate cells. This method, while not quantitative, reflects the presence of micrometastatic foci and, further, allows the possibility of studying the cell population that has invaded the lymph nodes.

Processing for Microscopic Analysis. Fragments of tumors and lymph nodes were placed directly into Tissue-Tek embedding medium (O.C.T. compound; Miles), frozen in isopentane, cryosectioned, and stored at −80°C until processed for β-galactosidase-specific staining. Alternatively, parts of the tumors were fixed in Bouin's solution, embedded in paraffin, and sectioned for anatomopathological microscopic analysis.

RESULTS

Tumorigenicity of NBT-II Carcinoma Cells. We first looked at the tumorigenicity of NBT-II cells in nude mice. It is well known that tumor induction in nude mice depends critically on cell number and cell type (16). NBT-II cells were tumorigenic when 5×10^6 cells were injected subcutaneously into nude mice, and after a long latency period, the tumor cells metastasized to the lymph nodes (Table 1). The same response was obtained with 3.5×10^6 cells (Fig. 1) but inoculation of 2×10^6 cells was not sufficient, as tumor grafts were unsuccessful even after 10 weeks of latency.

Production of aFGF by the NBT-II Carcinoma Cells Enhances Their Tumorigenic and Metastatic Potentials. To determine whether carcinoma cells producing a growth factor posses a tumorigenic and/or enhanced metastatic advantage, we tested the *in vivo* behavior of a series of aFGF-producing sublines. One clone (NSF14) constitutively produces 166 ng of aFGF per 10^7 cells (12). aFGF was detected by immunoreactivity in the extracellular matrix and was not found free in the conditioned medium of the cells (12). *In vitro* proliferation of both NSF14 and NBT-II cells was almost equivalent in 10% (vol/vol) fetal calf serum-containing medium (data not shown). *In vivo* NSF14 cells behaved differently from NBT-II cells in that 3.5×10^6 cells induced large tumors of 2–3 cm^3 within 2 weeks after injection. As mentioned for control NBT-II cells, a threshold cell number to be injected efficiently was observed. Again, subcutaneous inoculation of $<3.5 \times 10^6$ cells was not efficient for tumor grafting in this system, no matter what amount of growth factor was produced, at least in the range tested. Tumor growth was not a function of growth factor production *per se* since NSF14 and other aFGF-producing clones gave rise to the same proliferative tumors with identical growth rates, although their levels of aFGF synthesis varied from 4.5 to 166 ng per 10^7 cells (Fig. 1). Analysis of 14-day-old tumor sections clearly indicated that the rapidly growing tumors obtained with the aFGF-producing cells were very deficient in host stromal cells compared to 9-week-old control NBT-II carcinomas (data not shown). Micrometastases were detected in the lymph nodes as early as 2 weeks after inoculation of the NSF14 cells (Table 1), and all the animals were sacrificed at 14–16 days.

Table 1. Frequency of lymph node micrometastases in nude mice injected with 3.5×10^6 epithelial NBT-II cells or aFGF-producing cells (NSF14 cells)

Time after injection, weeks	% lymph node micrometastases	
	NBT-II cells	NSF14 cells
2	0 (0/8)	33 (10/30)
5	23 (3/13)	–
8	22 (2/9)	–
9	75 (3/4)	–
12	66 (2/3)	–

Number of animals bearing micrometastases/total number of injected animals is given in parentheses.

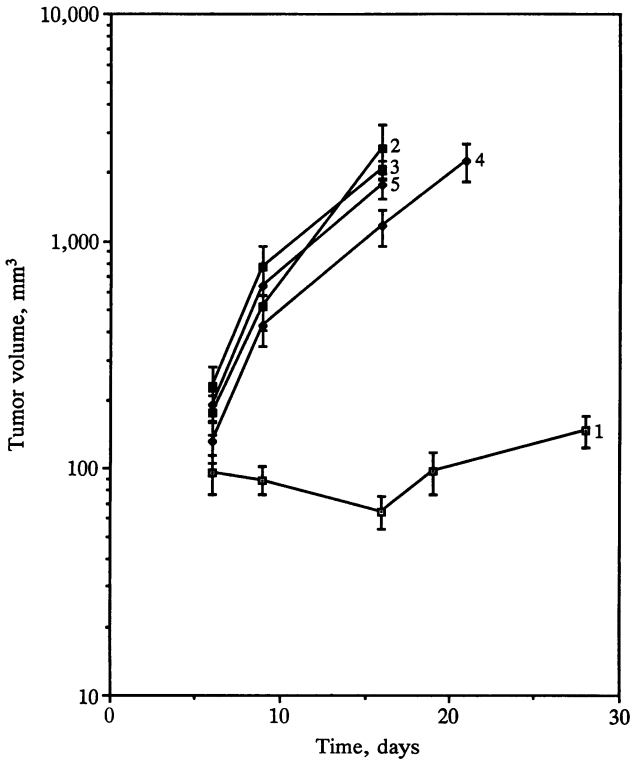

FIG. 1. Comparative tumor proliferation obtained with various aFGF-producing clones. NBT-II cells producing the human aFGF were obtained by transfection as reported (12). Their respective *in vitro* production is as follows. Curves: 1, NBT-II, no production of endogeneous aFGF; 2, NSF13, 4.5 ng per 10^7 cells; 3, NSF14, 166.7 ng per 10^7 cells; 4, NSF32, 13.1 ng per 10^7 cells; 5, NSF46, 10.7 ng per 10^7 cells. Each nude mouse was injected s.c. with 5×10^6 cells. Results represent the mean tumor volume of five animals, and the bars are the SEM.

aFGF-Producing Cells Confer Their Advantage to the Whole Carcinoma. We next questioned whether NSF14 cells can confer their advantage to a whole carcinoma. To do so, we simulated tumor heterogeneity by mixing growth-factor-producing cells with autocrine-negative counterparts. Tumor formation and metastatic spread were induced in nude mice by s.c. inoculation of NBT-II/NSF14 mixed cell populations. To define the cell origin within the primary tumor and the corresponding metastases, NBT-II cells transfected with the reporter β-galactosidase gene (β-gal–NBT-II cells) were coinjected with NSF14 cells identifiable by the presence of the human aFGF sequence. Swiss nude mice were coinoculated with a constant number of a 3.5×10^6 cells as a cell

mixture consisting of β-gal–NBTII cells with decreasing amounts of NSF14 cells at ratios from 1:7 (14.3%) to 1:3500 (0.028%). Results illustrated in Fig. 2 show that latency for tumor appearance (200 mm^3) is 5 days for NSF14 cells, 35 days for NBT-II cells, and 20 days for a tumor cell mixture containing NSF14/NBT-II cells at a 1:3500 ratio. Tumor growth curve slopes were very similar for tumor obtained with all the cell mixtures (Fig. 2*A*) and the latency period was significantly dependent on the NSF14 cell ratio in the mixture (Fig. 2*B*), suggesting that the main effect of the presence of aFGF-producing cells is the shortening of the lag time before tumor appearance rather than the effect on tumor growth by itself. Within 2–3 weeks, micrometastases in the lymph nodes were identified by growth *in vitro*. Micrometastases have been detected for the different carcinoma cell mixtures when the tumor size reached ≈200 mm^3, in agreement with the generally accepted notion that occurrence of metastasis correlates with tumor size. Therefore, our data suggest that the early appearance of micrometastases correlates with the ratio of aFGF-producing cells in the inoculate (Fig. 2).

Are Growth-Factor-Producing Cells Dominant *in Vivo* for Tumor Progression? To address the possibility of dominance by the growth-factor-producing cells, the phenotype and genotype of the tumor cells and of the lymph nodes obtained after injection of the various cell mixtures were analyzed. β-galactosidase activity was detected in all tumors obtained after injection of mixed cells in an amount that reflected the percentage of β-gal–NBT-II cells injected: 86–90% of the primary-tumor-derived cells grown in culture were β-galactosidase-positive, when nude mice were injected with a mixture containing 0.5 × 10^6 NSF14 cells and 3 × 10^6 β-gal–NBT-II control cells. Twenty-five days after inoculation, the axillary lymph nodes of the mice injected with NSF14/NBT-II cells in a 1:3500 ratio contained tumor cells that were almost entirely positive for β-galactosidase activity. *In vitro*-growth explants of the tumor and of the lymph node-invading cells gave similar results, demonstrating that the metastases are not monoclonal in that they contain both NBT-II and NSF14 cells. Staining for β-galactosidase activity in the sections of lymph nodes indicated that numerous β-gal–NBT-II cells developed independent foci; similar results were obtained in 80% of the animals sacrificed 35 days after inoculation of a mixture containing 10^3 aFGF-producing cells, leading to the same conclusion (Fig. 3). Southern blot analyses revealed the presence of both β-galactosidase and aFGF sequences in the tumor and the corresponding metastases (data not shown).

DISCUSSION

We have shown (12, 13) that NBT-II carcinoma cells designed to produce aFGF after transfection acquired invasive properties in *in vitro* experiments. We here demonstrate that the tumorigenicity of NBT-II carcinoma cells increases when they produce aFGF, and we further tested the hypothesis that even where only a minority of tumor cells become committed to produce their own growth/dissociating factor, the tumor will progress to acquire invasive and metastatic potential. Furthermore, if this does occur within a carcinoma, the questions are, do the aFGF (or another growth/dissociating factor)-producing cells escape from the tumor mass and become the invasive and metastatic subpopulation or do they confer metastatic capacity on cells that do not produce the factor as well?

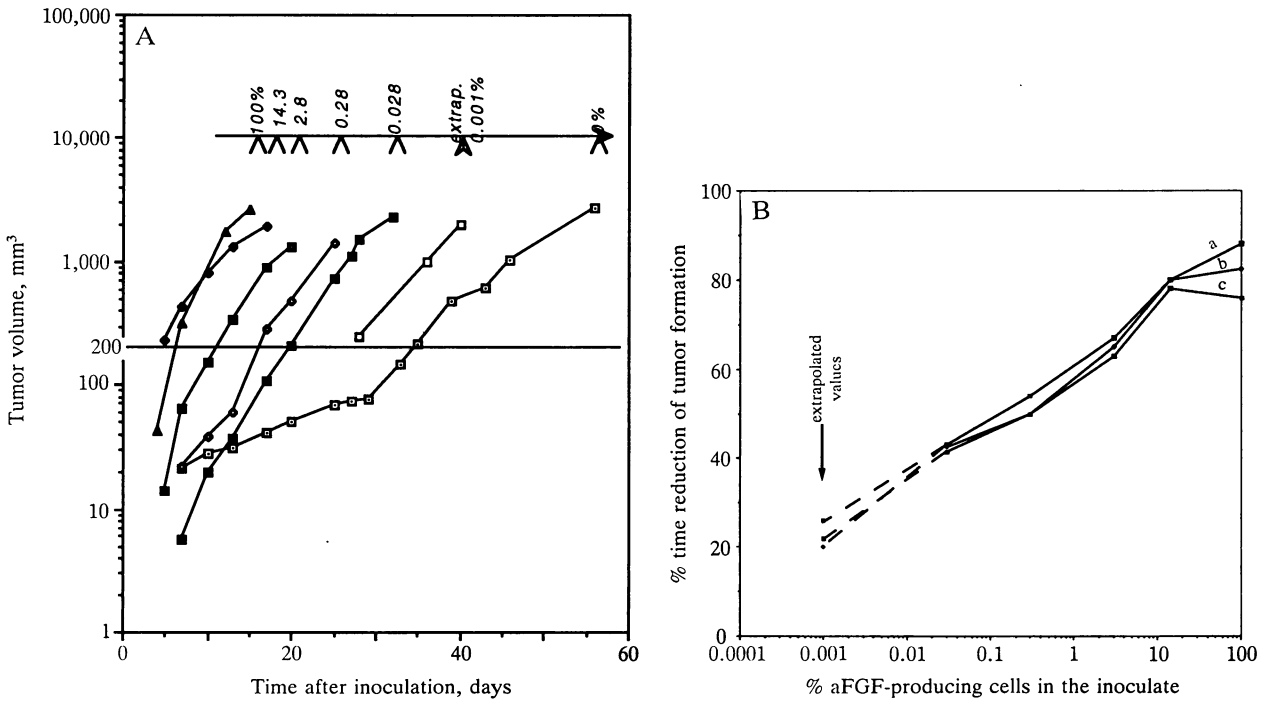

FIG. 2. Tumor proliferation after injection of mixed cell populations. (*A*) Proliferation of tumors obtained after coinjection into nude mice of a constant number of cells (3.5 × 10^6 cells) consisting of a decreasing percentage of NSF14 cells relative to NBT-II cells. NBT-II cells were injected s.c. into 6-week-old female Swiss nude mice (*nu/nu*). Each coordinate corresponds to the mean of the tumor volume of five mice; for NBT-II cells alone (0%) and NSF14 alone (100%), the coordinate is the mean of two experiments, each with five animals. Coordinates corresponding to 0.001% are extrapolated (extrap.). Extrapolation was obtained by taking into account the shortening of lag time for a tumor volume of 250 mm^3, 1000 mm^3, and 2000 mm^3 vs. the ratio of NSF14 cells in the mixture injected: 14.3%, 500,000 NSF14 cells; 2.8%, 100,000 cells; 0.28%, 10,000 cells; 0.028%, 1000 cells; extrapolation 0.001%, 35 cells. (*B*) Reduction in the delay of tumor appearance as a function of proportion of aFGF-producing cells. Coordinates represent the percentage of time reduction for tumor formation deduced from *A* of 200-mm^3 (curve a), 500-mm^3 (curve b), and 1000-mm^3 (curve c) tumors. Extrapolation of these curves indicates a shortening of 20–26% for 0.001% of NSF14 cells (i.e., 35 of 3.5 × 10^6 cells) in the mixture.

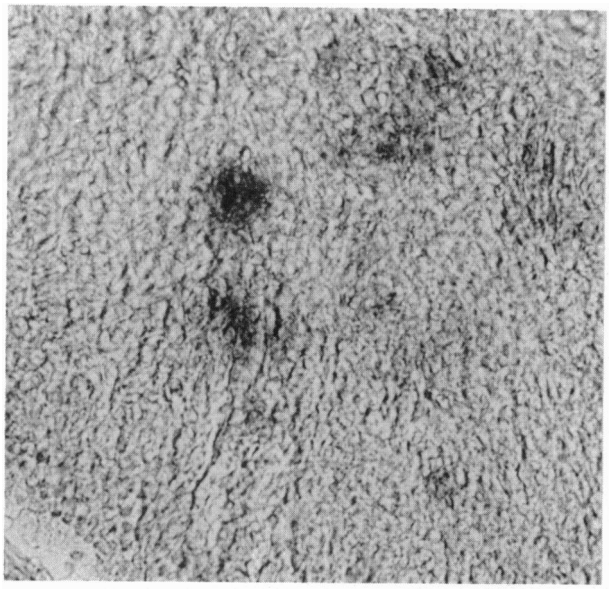

FIG. 3. β-Galactosidase staining of a lymph node section. Axillary lymph node of a nude mouse 35 days after injection of a cell mixture containing 0.028% of aFGF-producing cells was fixed with paraformaldehyde/glutaraldehyde, stained for β-galactosidase, and counterstained with eosin. Control staining experiments with lymph node sections of a normal nude mouse or of nude mice injected with cells that do not express β-galactosidase are negative (data not shown). (×155.)

Our data unambiguously show that various cell mixtures mimicking heterogeneous cancer cell populations, including potentially "committed" aFGF-producing cells, are globally highly tumorigenic and subsequently metastatic in nude mice. Cells producing aFGF do not have an *in vivo* proliferative advantage nor an enhanced metastatic behavior over the nonproducing cells in the primary tumor, and the mixed populations behave more like the aFGF-producing cells alone than like the nonproducing cells. We exclude the possibility that clonal dominance may arise later in this system as the animals have developed large heterogeneous tumors (≈2 cm^3) and display lymph nodes invaded with both cell types. These results suggest that aFGF-producing cells do not drive tumor progression by dominance but rather confer their potential to neighboring cells by autocrine or juxtacrine cooperativity. In this model aFGF-producing cells appear to have a leader effect for tumor proliferation and, consequently, for metastatic behavior. The autocrine or juxtacrine effect of aFGF has to be considered as acting directly through signaling or indirectly through the induction of other factors that remain to be identified. In any event, all tumor cells adopt an *en bloc* behavior.

Extrapolation to the *in vivo* growth of a tumor cell population containing as few as 0.001% of aFGF-producing cells (Fig. 2) indicates a significant reduction of 20–26% for the delay of appearance of a 200-, a 500-, or a 1000-mm^3 tumor (26 days vs. 35, 32 days vs. 41, or 36 days vs. 46, respectively); shortening this delay is a function of the ratio of NSF14 cells and likely of the quantity of aFGF produced. *In vitro* NSF14 cells produce a large amount of aFGF (166 ng per 10^7 cells) and part of it can be sequestered in the extracellular matrix (12). The possible mechanism of release of the growth factor *in vivo* is not yet elucidated but recently several hypotheses have been proposed, including cell leakage, cell death, sublethal cell injury, or a still unknown secretory pathway; it remains an open question whether FGF needs to be released to be functional or can act intracellularly or in a membrane-bound form (17).

Tumor-cell interactions between genetically distinct subpopulations existing within tumors have begun to be investigated (18). It has been shown with experimental *in vitro* or *in vivo* models that these interactions could take place through diffusible mediators, such as growth factors (19–21) or proteases and their inhibitors (22), or through cell-contact-mediated mechanisms (23, 24). More, tumor-cell interactions with the host cells are of crucial importance and stromal influences are documented (25–28).

Another type of intimate tumor–host interaction is the setting up of the tumor neovascularization that can be induced through soluble factors (29, 30) such as aFGF and basic FGF (30, 31). It is thus tempting to postulate that in addition to its already mentioned activities, aFGF produced by the NSF14 cells may account for the rapid proliferation of carcinoma cells by facilitating the prompt neovascularization in nude mice.

The *in vivo* model system described here shows that among two cell populations of the same origin, both populations expressing the corresponding high-affinity receptors but only one population producing aFGF, there is no clonal dominance either for tumor proliferation or for metastatic spreading but rather a community effect as reported for biological situations other than neoplasia (32) via direct or indirect aFGF signaling.

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