Prolonged Dormancy and Site-specific Growth Potential of Cancer Cells Spontaneously Disseminated from Nonmetastatic Breast Tumors as Revealed by Labeling with Green Fluorescent Protein

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ABSTRACT

This study used an isogenic pair of metastatic (M4A4) and nonmetastatic (NM2C5), green fluorescent protein-labeled human breast cancer cell lines derived from the same patient and inoculated into the mammary glands of nude mice to investigate the dissemination patterns and fate of cells that escaped spontaneously from the resulting tumors. After tumors appeared, fluorescing single tumor cells were regularly seen in the lungs, even in animals inoculated with NM2C5, which fails to form secondary tumors in other organs. The sensitivity of the technique confirmed the continuing presence of scattered NM2C5 cells after primary tumor resection, although they formed no metastases by 6 months. These self-disseminated human tumor cells were retrievable from the tissues and were still viable and malignant, manifested by indefinite proliferation *in vitro* and green fluorescence and local tumorigenicity *in vivo*. Therefore, these scattered tumor cells were still immortal but rendered indefinitely quiescent by the microenvironmental conditions in the lung tissue. This is the first unequivocal demonstration of spontaneous distant dissemination of human cancer cells by undisturbed nonmetastatic tumors and comprises a valuable system for the analysis of tumor dormancy. In contrast, although many of the cells disseminating from M4A4 tumors grew into fluorescing metastases in the lungs, others remained solitary and quiescent. Therefore, even in a clonally derived cell population with metastatic properties, many cells do not, or cannot, mobilize the organ-specific growth properties needed to generate metastases. This experimental approach, by using self-disseminating, green fluorescent protein-labeled, sister cell lines of opposing metastatic phenotypes, opens new avenues for investigating topics of clinical relevance, including tumor cell dormancy, anatomical distribution of metastases, and host factors influencing the metastatic process.

INTRODUCTION

Since the time of Paget (1), investigators have struggled to understand the mechanisms by which tumors metastasize and how the distribution of tumor deposits is not random but linked to the site of origin of the primary tumor. As the disseminating tumor cells are freely and ubiquitously distributed by the body fluids, it is curious that they establish secondary tumors in a predictable pattern of distant organs but do not do so in many others in the same individual. It is also notable that such scattered cells can sometimes remain dormant in the patient's tissues for long after the primary tumor was excised but can later reactivate growth. These repeatedly validated clinical and pathological observations confirm that migrating tumor cells do not indiscriminately make secondary tumors wherever they land (2, 3).

From this, it follows that tumor cell dissemination by hematogenous, lymphatic, or *trans*-coelomic routes is necessary but not sufficient for the formation of secondary tumors and that, unless a growing tumor colony is established in a new site, the metastatic process is not fulfilled by that cell. Mechanistically, it has long been inferred from observations on humans (1, 4) and animals (5) that the local organ microenvironment at the site where the tumor cells come to rest influences whether metastases emerge or not. The nature of these influences deserves investigation to determine whether they can be manipulated for therapeutic benefit.

In this communication, we report a study using cloned human tumor cell lines of diametrically opposite metastatic performance (6, 7), which we labeled with GFP[2] to visually track disseminating tumor cells of differing malignant potentials and examine their fate in different host microenvironments. Additional objectives of this work were to ascertain whether such cells die within a few days, if they fail to grow in the ectopic organ sites where they implant, and to retrieve disseminated cells which are not making metastases, so as to assess their viability, proliferative potential, and tumorigenicity. The study was also conducted to ascertain whether these human breast cancer cells make bone metastases in nude mice.

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[2] The abbreviations used are: GFP, green fluorescent protein; mfp, mammary fat pad.

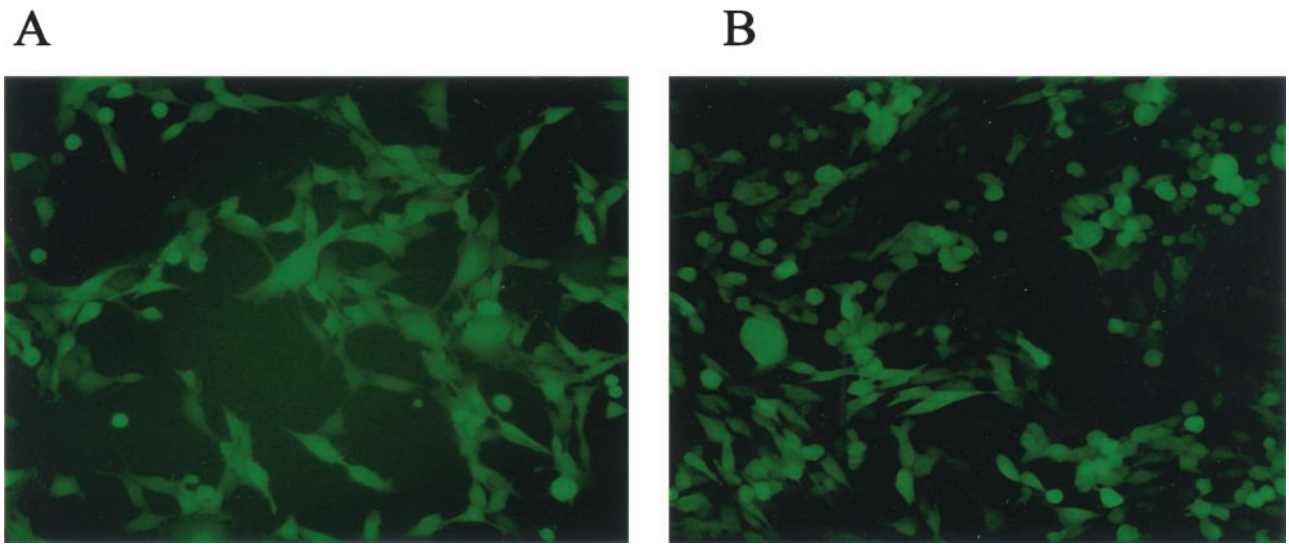

Fig. 1 Stable, uniform high level GFP expression of human breast cancer MDA-MB-435 cells. Subclones of the cell line were transduced with the pLEIN retroviral vector that expresses enhanced GFP and the neomycin resistance gene. Stably transduced lines were selected with G418. The cell populations remained uniformly fluorescent over several weeks in culture. *A*, nonmetastatic NM-2C5 clone. *B*, the metastatic M-4A4 clone. Magnification: ×400.

One of the main aims of this work was to focus on the spontaneous, unprompted dissemination of labeled cancer cells from an undisturbed tumor and their subsequent colonization of distant organs or dormant residence there. Some previous studies (8–10) have inoculated GFP-labeled cells directly i.v. to study their short-term fate, but there has been no previous inquiry tracking the behavior and fate of cells escaping from primary tumors by themselves over prolonged periods of time. An additional novel feature in the design of the current work was to compare the behavior of GFP-labeled tumors, which are spontaneously metastatic, with that of labeled tumors formed by an isogenic sister cell line, which is virtually unable to make secondary tumors in distant organs. Detailed characteristics of this pair of cell lines have been described previously (7). The present study yielded unexpected new information showing that the tumors which could not make detectable secondary tumors in any organ, were nevertheless shedding tumor cells into the circulation. The genetically incorporated label showed that these remained solitary and dormant in the tissues for months. In addition, it was proven that these cells could be retrieved and would resume proliferative capability *in vitro* as well as tumorigenicity when reinoculated into the mammary gland.

MATERIALS AND METHODS

GFP Expression Vector and Retroviral Production. The RetroXpress pLEIN vector (Clontech, Palo Alto, CA) contains both enhanced GFP and the neomycin resistance gene expressed via a bicistronic message that contains an internal ribosome entry site (11–16). The PT67 packaging cell line (Clontech) was cultured in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.). For retroviral vector production, PT67 cells were incubated at 75% confluency with a precipitated mixture of DOTAP reagent (Roche Molecular Biochemicals, Indianapolis, IN) and saturating amounts of pLEIN vector for 18 h. Medium was replenished, and transduced cells were selected by culture in the presence of 200-1000 μg/ml of the neomycin analogue G418 (Life Technologies, Inc.) for 7 days.

Retroviral Transduction and Selection of GFP-expressing Tumor Cell Lines. To achieve GFP gene transduction into the M-4A4 and NM-2C5 clonal tumor cell lines, 20% confluent cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and RPMI 1640 (Life Technologies, Inc.) containing 7% fetal bovine serum (Life Technologies, Inc.) for 72 h. After media replenishment, M-4A4 and NM-2C5 cells were harvested by trypsin-EDTA 72 h post-transduction and subcultured at a ratio of 1:15 into selective medium that contained 200 μg/ml G418 initially. The level of G418 was subsequently increased to 800 μg/ml stepwise. Clones expressing GFP (NM-2C5-GFP and M-4A4-GFP) were selected, combined, and propagated using conventional culture methods. To evaluate the doubling time of GFP-labeled clones, cells were seeded at 1.5×10^4 in 35-mm culture dishes, harvested, and counted using a hemocytometer every 24 h.

Orthotopic Inoculation and Primary Tumor Growth. Female athymic mice (BALB/c nu/nu; B&K Universal, Fremont, CA) aged 8–10 weeks were housed in an isolation suite for the duration of the experiments and cared for in accordance with the standards of University of California San Diego under an approved protocol of the University of California. The tumorigenicity and spontaneous metastatic capability of the cell lines were determined by orthotopic inoculation into the mfp. One million cells in 0.05 ml of a 1:1 mixture of RPMI 1640 and extracellular matrix gel (Sigma Chemical Co., St. Louis, MO) were inoculated into the surgically exposed right flank mfp of an anesthetized mouse under direct vision through a dissecting microscope. This ensured delivery of the entire inoculum into

the desired location. Animals were monitored every 2 days for ≤5 months for tumor growth and general health. The rate of primary tumor growth was determined by plotting the means of two orthogonal diameters of the tumors, measured at 7-day intervals. Animals were euthanized and autopsied at 3–6 months postinoculation, the autopsy being performed when a primary tumor reached a diameter of ~2 cm. For analysis of experimental metastases, BALB/c nu/nu mice were injected with 1×10^6 cells via the tail vein. Cells were harvested using EDTA and injected in a total volume of 50 μl of PBS. Mice were euthanized 6 weeks after inoculation and examined as described below.

Evaluation of Spontaneous Metastasis Distribution, Tumor Cell Dissemination, and Cell Survival. At autopsy, the whole animal, the primary tumor, and all of the major organs were examined under blue light of wavelength 490 nm to activate GFP fluorescence using a commercial blue light fiber optic rig (Lightools Research, Encinitas, CA). Images were captured on a thermo-electrically cooled color digital camera fixed on an adjustable rig, and the collective ensemble will be referred to as the GFP imaging system. Small foci of GFP-expressing cells detected on the dorsal surface of the left upper lobe of the lung were counted. Excised tissues were stored for subsequent analysis after either fixation in 10% neutral formalin or snap freezing in liquid nitrogen. For visualization of GFP-labeled cells within fixed lung tissue, the left upper lobe of the lung was perfused with OCT compound (Sakura, Torrance, CA), and 50-μm frozen sections were viewed immersed in saline. Light and fluorescence microscopy was carried out using a Nikon E600 microscope equipped with a GFP filter set (Chroma Technology, Brattleboro, VT) and a xenon lamp power supply. Images were processed by Metamorph Version 3.0 software (Universal Imaging Corp., Downingtown, PA).

Tumor Excision and Cell Retrieval Experiments. To evaluate whether a concomitant primary tumor inhibits the growth of metastases in this experimental system, the tumors were surgically removed at 1 cm in diameter from 10 orthotopically inoculated mice, with subsequent autopsy 3 months later. At autopsy, surface metastases and disseminated labeled cells were counted. Recovery of viable GFP-labeled cell lines from the lungs of NM-2C5 inoculated animals was accomplished by culture of tissue explants. Lung tissue was crudely homogenized by scalpel and cultured in RPMI 1640 (Life Technologies, Inc.) containing 10% fetal bovine serum (Life Technologies, Inc.) and penicillin/streptomycin. After 72 h, debris was removed, and adherent cells were subcultured in selective medium that contained 200 μg/ml G418. The concentration of G418 was subsequently increased to 800 μg/ml stepwise over 4 weeks of culture. Viable recovered cells at this time were still GFP positive.

RESULTS

Stability of GFP Expression in Labeled Breast Tumor Cells. Retrovirally transduced NM-2C5 and M-4A4 breast tumor cells containing the GFP and neomycin resistance genes were able to thrive *in vitro* at concentrations of G418 ≤800 μg/ml. The selected cells maintained uniform bright GFP fluorescence in the whole population (Fig. 1) during continued passage after G418 selection was completed, indicating that the fluorescence was sufficiently stable to permit detection after inoculation and long-term growth *in vivo*. The proliferation rate of the transduced cells was not altered by the transduction procedure or by GFP expression, as evidenced by the comparison of the doubling times with the nontransduced cell lines (data not shown). The distinct morphology of the NM-2C5 and M-4A4 clones was also unchanged by the transduction procedure, with NM-2C5 cells displaying a characteristic pattern of growth in tightly clumped aggregates (7).

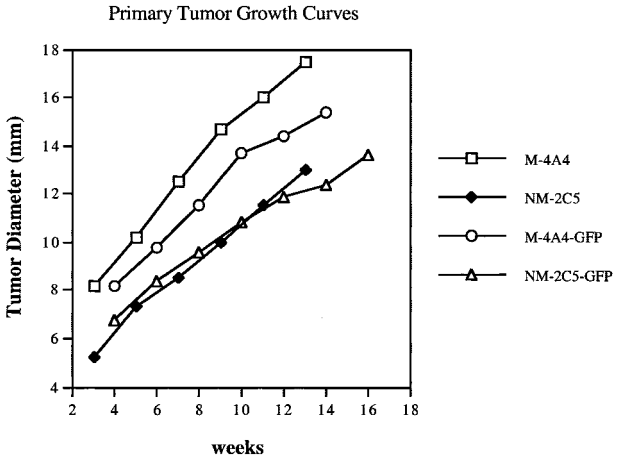

Fig. 2 Growth rates of primary tumors resulting from inoculation of GFP-labeled or unlabeled breast tumor cells into athymic mice. The rate of primary tumor growth was determined by plotting the means of two orthogonal diameters of the tumors, measured at 7-day intervals.

Growth Characteristics of GFP-expressing Primary Breast Tumors. Primary tumors formed in the mfp were palpable 2 weeks after orthotopic inoculation, and progression was monitored until euthanasia when tumors had reached ~2 cm in size. All inoculated animals developed mammary tumors and remained active and strong until sacrifice. The growth curves of the tumors formed by the two cell lines were compared with each other and to the growth of tumors formed by the parental cell lines by weekly orthogonal measurements (Fig. 2). The observation described previously that the metastatic M-4A4-derived tumors grow faster than their NM-2C5 counterparts (6, 7) was confirmed. The growth rate of primary tumors formed by both of the GFP-transduced cell lines was broadly similar to that of unlabeled tumors (Fig. 2), indicating that the behavior of the lines was not dramatically changed by the transduction procedure. NM-2C5-GFP primary tumors took between 112 and 191 days (mean 155 days) to reach the experimental end point of 2-cm tumor diameter in one plane of measurement. M-4A4-GFP tumors took between 97 and 143 days (mean 113 days) to reach the same end point size (Table 1). In all cases, the tumor tissue was strongly fluorescent (Fig. 3), demonstrating stable high level GFP expression by the constituent cells throughout extended periods of growth *in vivo*.

Metastatic Phenotype of GFP-labeled Xenografts. At autopsy, the intact body was first examined with the GFP imaging system. The intensity of GFP fluorescence allowed the primary tumor to be clearly seen transcutaneously (Fig. 3).

Table 1 Tumorigenicity and production of metastasis by GFP-labeled MDA-MB-435 clones orthotopically implanted in athymic mice

Cell Line	No. mice	Mean days postinoculation (range)	Mean diam of primary tumor (cm) (range)	No. animals with lung metastasis	Median lung surface metastatic deposits (range)	Mean no. of isolated cells visible on lung surface
M-4A4-GFP	8	113 (97–143)	2.0 (1.8–2.2)	5/8 (62%)	4 (1–9)	73
NM-2C5-GFP	9	155 (112–191)	1.7 (1.5–1.9)	0/9	0	50
NM-2C5-GFP with resection of primary tumor	9	239 (208–271) 89–96 days postresection	1.8 (1.6–2.0)	0/9	0	5

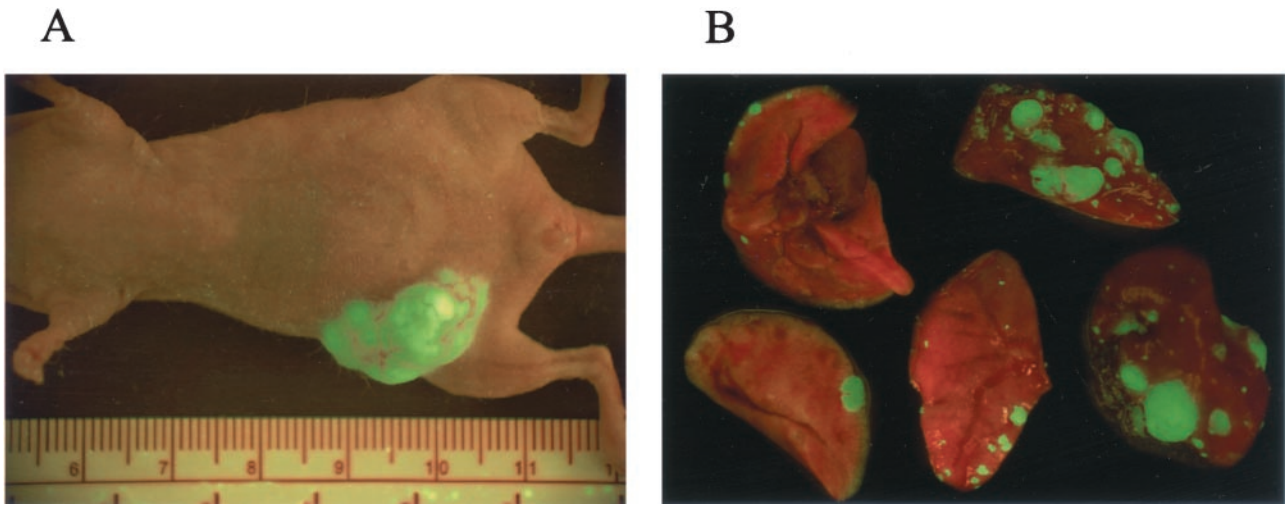

Fig. 3 Orthotopic growth and lung metastases visualized by GFP. *A*, a primary tumor of M-4A4 cells formed in the mfp visualized under blue light. *B*, metastases of M-4A4 cells visualized in the excised lungs of the nude mouse. Magnification: ×10.

Although previous studies (16) have unequivocally demonstrated that internal lesions can be detected if they are large enough and close enough to the skin of the animal, internal metastases were not visible under blue light illumination in this study. On opening the body, the surface of all of the internal organs was first examined under ordinary illumination. Low magnification microscopy revealed easily detectable macroscopic lung metastases in 5 of 8 (62%) mice inoculated with M-4A4-GFP cells (Table 1). Subsequent examination under blue light (490 nm) showed that all metastatic deposits detected by light microscopy exhibited green fluorescence characteristic of GFP-labeled tumor cells. Metastases were not seen in any other organs in mice bearing 4A4 tumors within the time period of this experiment, although they do occur frequently in lymph nodes (7).

Conversely, tumors derived from the clonal cell line NM-2C5 did not produce any visible metastases in the host lungs, nor in any other organ (Table 1). However, microscopic examination of the surfaces of the lungs and of frozen sections of these organs under blue light revealed the unequivocal presence of many disseminated tumor cells that were invisible by conventional illumination. The most striking observation was that these were clearly present in the lungs of all animals which harbored tumors formed by NM-2C5 cells in their mammary glands, as well as in those with tumors formed by M-4A4 cells. Despite the intense fluorescence of these single tumor cells, it was impossible even at the highest magnifications to establish with certainty whether they had exited from the capillaries into the lung parenchyma, because precise visualization of the capillary endothelium within the lung was not feasible. Tiny tumor cell emboli of one to four cells, which would be undetectable macroscopically or even microscopically, were easily located because of their fluorescence (Fig. 4).

The viability of the disseminated NM-2C5 cells that survived in the murine lung tissue was demonstrated by the recovery, in drug selection medium, of a proliferating GFP-labeled cell line (named NM-2C5L) cultured from lung explants. We considered it formally possible that NM-2C5 cells which had arrived, and survived in the lung environment, were ones that had an invasive or growth advantage or that they could have been conditioned by their stay in the lungs to be able to form metastases if reinjected *in vivo*. We, therefore, tested this by inoculating these cells (NM-2C5L) into athymic mice via the tail vein. The tail vein route delivers a large bolus of cells to the lung, being the first capillary bed encountered. This assay is a more direct test of the ability to survive and/or proliferate in the lung environment because the invasive and migratory steps in spontaneous metastasis are circumvented. With this form of inoculation, fluorescing micro-emboli of NM-2C5L cells were found in the lungs of three of five mice inoculated, but no experimental metastases were evident. Fluorescent s.c. primary tumors grew around the injection site in two of these animals.

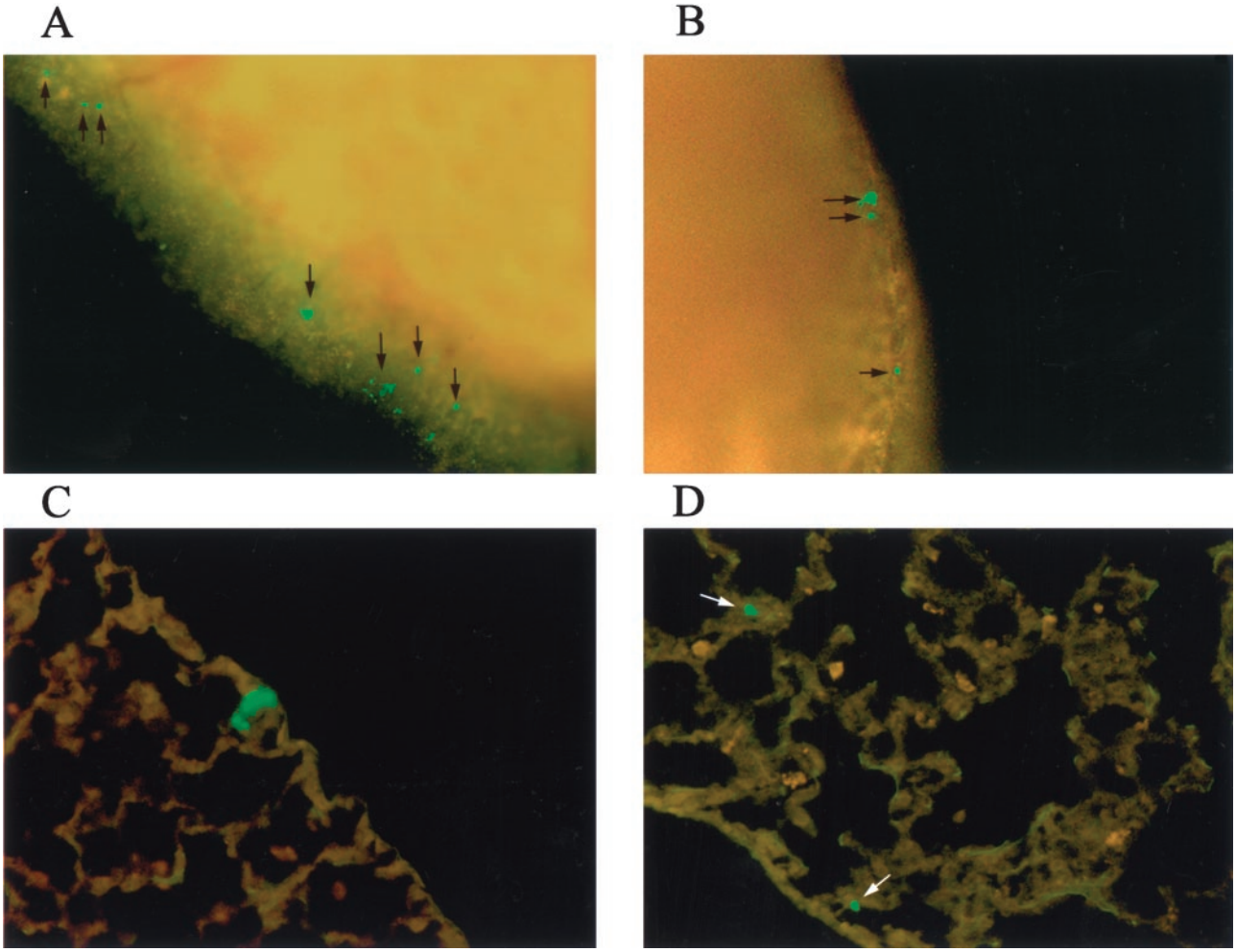

Fig. 4 Solitary GFP-labeled tumor cells (*arrows*) and microemboli present in the lungs of BALBc/nu/nu mice. *A*, lung surface revealing GFP-labeled M-4A4 cells. Magnification: ×40. *B*, lung surface displaying NM-2C5 cells. Magnification: ×40. *C*, lung section from NM-2C5-inoculated mouse. Magnification: ×100. *D*, lung section 3 months after NM-2C5 primary tumor removal. Magnification: ×100.

Additionally, reinoculation of this new cell line into the mfp of three additional animals resulted in local tumors in all of them (three of three), confirming the tumorigenicity of the cell inoculum, but no metastases occurred in the lungs in any mouse.

Because of the fact that breast cancer in humans is often observed to spread to the long bones and the axial skeleton, we particularly wanted to investigate whether the cell lines that we have cloned also metastasize to these organs. GFP fluorescence greatly facilitates this analysis; metastases in skeletal sites have been revealed previously in other models of metastasis (13–15), with bone metastases being detectable through the skin of nude mice (16). Fluorescent examination revealed no skeletal tumor deposits in mice inoculated with either metastatic M-4A4 or nonmetastatic NM-2C5 cells. In some cases, the skeleton was cleaned of all soft tissue before illumination, but still no metastases were revealed.

Effects Of Primary Tumor Excision on Disseminated NM-2C5 Tumor Cell Dormancy. These experiments were conducted to establish whether a concomitant primary tumor in the mammary gland can suppress metastasis formation by disseminated NM-2C5 cells. The primary tumor was removed at a size of ~1 cm, after an average of 16 weeks growth, and the mice were euthanized 3 months later. No metastases were found in any of the organs of nine animals from which the primary tumors were excised (Table 1). However, the survival and distribution of solitary brightly fluorescing cells in the surface of the lungs were confirmed, although the numbers were diminished relative to those in mice which harbored a primary tumor throughout the experiment (Fig. 4 and Table 1), presumably because of excision of the source from which they had been seeded. Viable cells were also recoverable in culture from the lungs of these mice showing that, although unable to proliferate, NM-2C5 cells can remain viable in the lung environment for ≤6 months.

DISCUSSION

This work clearly demonstrates the survival, viability, and recoverability of spontaneously disseminated, quiescent breast

cancer cells, which had not formed metastases in the lungs or any other organs of the host. These cells had remained dormant in the tissues for extended periods of time, equivalent to a quarter of the lifetime of the host. Their failure to form metastases was not attributable to any intrinsic loss of proliferative capacity, as demonstrated by the ability of the cells to grow exponentially and indefinitely when retrieved from the lung tissue and cultured *in vitro* or reinoculated into the mammary gland. It was also not attributable to inhibition by circulating factors from a concomitant primary tumor in the mammary gland, as indicated by the absence of metastases after resection of the primary tumor, although tumor cells could still be retrieved from the lungs and grown *in vitro*.

These findings establish definite biological distinctions between metastasis, tumor cell dissemination, and tumor cell dormancy in this experimental system, which may apply more generally. They also indicate that the mitotic quiescence of disseminated NM-2C5 cells is induced by the surrounding host cells, although their ancestral counterparts, which they leave behind, grow progressively in the host's mammary gland. Indeed, the very same disseminated cells, retrieved from the lungs, retain their tumorigenicity when reinoculated back into the mammary gland. The implications of this are that cells with unlimited proliferative potential, such as tumor and stem cells, are not invincible and can be returned to a state of suspended mitotic activity by the surrounding cellular microenvironment.

It is appropriate to refer here to previous investigations (8–10), which have described the presence of viable tumor cells that did not form tumor deposits in downstream organs after i.v. inoculation of labeled cells. The retrieval of such experimentally seeded cells, after periods of 1–30 days, and their ability to grow *in vitro* have also been reported (10). However, so far as we are aware, the present article is the first account of the identification and retrieval of labeled, viable, human tumor cells, which had spontaneously disseminated themselves from an undisturbed tumor and lain dormant in the host for several months. The dissemination kinetics of such cells, escaping over prolonged periods of time, are clearly different from those in a pulse bolus inoculated into a large vein, and this experimental design provides the new data, unobtainable by other methods, that tumors (*e.g.*, NM2C5), which do not establish distant secondary deposits, can still be shedding neoplastic cells into the vascular system. The present data are also novel because they show, for the first time, that these naturally disseminated, dormant tumor cells can still proliferate indefinitely and generate tumors in appropriate circumstances. The current study, therefore, confirms and extends these (10–16) and other (17, 18) previous reports and offers new opportunities for the investigation of factors that affect the growth of spontaneously disseminated tumor cells in distant organs. It also documents a new model for the experimental analysis of conditions causing long-term dormancy of tumor cells that have escaped from an undisturbed tumor to other tissues.

It is worth noting that many of the cells disseminating from M4A4 tumors grew into fluorescing metastases in the lungs, although adjacent sister cells from the same tumor remained solitary and quiescent. Therefore, even in a clonally derived cell population with metastatic properties, many cells do not or cannot mobilize the organ-specific growth properties needed to generate metastases. These observations closely match those on patients treated with peritoneo-venous shunts for intractable malignant ascites, because of inoperable abdominal tumors (4). That clinico-pathological investigation showed that tumor cells grew to form metastases in some organs but not others, although billions of viable tumor cells were infused into the systemic circulation over the lifetime of the patient. Some patients did not develop any metastases at all, although viable solitary disseminated tumor cells could be seen in the tissues of downstream organs. Others developed more metastases in organs already known to harbor deposits but not in additional organs, despite prolonged survival of the host. In these patients, the malignant cells in the ascitic fluid were clearly viable and tumorigenic, as manifested by their ability to form abundant local metastases in the peritoneal lining of the abdominal cavity and in certain specific organs in the patients after vascular dissemination. The similarity of results in the present investigation to these data indicates the potential clinical value of results from this xenogeneic experimental system.

In some tumor systems, such as the Lewis lung carcinoma, investigators (19, 20) have provided evidence that the existence of a concomitant primary tumor in the host inhibits the growth of metastases by the release of soluble factors into the serum. However, the experiments conducted in the present investigation did not show any such effect in the breast-derived tumor system currently under study when the tumors were resected. This can therefore be rejected as a potential explanation for the failure of the disseminated tumor cells to grow in ectopic sites.

The clinical implications of the collective findings of this study and opportunities presented by this new experimental approach, combining GFP labeling and clones of differing malignant phenotypes, together introduce fresh stimulus for the analysis of the molecular mechanisms involved in metastasis and for designing its clinical control.

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