Early Cell Motility Changes Associated with an Increase in Metastatic Ability in Rat Prostatic Cancer Cells Transfected with the v-Harvey-ras Oncogene

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ABSTRACT

The development of metastatic ability by cancer cells is a multifactorial process whose temporal events are complex and poorly understood. One step in the metastatic process may involve cell motility. Previous studies reported correlations between motility and metastatic ability. Whether this correlation, seen in cancer cells maintained for long periods of time, is an epiphenomenon developing late in the growth of the cancer as a selection artifact of continuous passage, or is critically required for the acquisition of metastatic ability is unknown.

To investigate the relationship between cell motility and the acquisition of metastatic ability, advantage was taken of recently developed DNA transfection methods for inducing high metastatic ability in initially low metastatic cancer cells. The Dunning AT-2.1 cell line, a clonal rat prostatic cancer cell line with low metastatic ability, was transfected with a plasmid containing the neomycin resistance gene alone or in combination with the v-Harvey-ras oncogene. A series of the transfected cells was isolated by limiting dilution. After the first in vitro passage following transfection, cells were inoculated into rats to characterize their metastatic ability. The same transfectants were simultaneously studied using our visual grading system of cell motility to study the early motility changes associated with newly acquired metastatic ability. The data demonstrate increased membrane ruffling, pseudopodal extension, and cell translation (translocation) in the v-H-ras-transfected cell lines with high metastatic potential.

INTRODUCTION

The molecular mechanisms regulating the increased motility of neoplastic cells, first reported by Virchow in 1863 (1) and first documented with time-lapse cinemicroscopy by Gey in 1940 (2) still remains one of the most important, yet poorly understood, aspects of invasive tumor growth (3–5). Certain nontransforming peptide growth factors such as epidermal growth factor (6, 9), platelet-derived growth factor (7), insulin (8, 9), fibroblast growth factor (9), and transforming growth factor β (10) cause transient, short lived, membrane ruffling on the edges of nonneoplastic cells in culture. The transformation of mammalian cell lines by means of DNA transfection with oncogenes such as ras and with the Rous sarcoma virus have been associated with both an increase in cell membrane ruffling (11) and an increase in metastatic ability (12–15). Cells transformed with ras exhibit morphological changes in the cell membranes resembling ruffles. These changes have been observed with the electron microscope (16) and represent prolonged, continuous, membrane ruffling when studied either visually with phase microscopy (9), with a micropore filter assay (17), or temporally with time-lapse cinemicroscopy. The product of the Kirsten murine sarcoma virus gene (p21) has been localized with immunofluorescence to these ruffling regions of the cell membranes (18). Finally, microinjection of the p21 protein product of the human H-ras gene into cultured rat fibroblasts induced membrane ruffling in a dose-dependent fashion (19).

We have combined time-lapse videomicroscopy and a visual grading system of cell motility (20) to correlate membrane ruffling, pseudopodal extension, and cell translation with metastatic ability in the Dunning R3327 system of rat prostatic adenocarcinomas (21). While there was a correlation between a variety of the cell motility parameters and metastatic ability in this large series of Dunning prostatic cancers (21), each of the highly metastatic sublines studied had been serially passaged over an extended period of time (i.e., >1 year) before being tested for their respective motility values. This raised the issue as to whether the correlation between metastatic ability and motility was an epiphenomenon of continuous serial passage or was fundamental to the process of metastasis.

To answer this question more directly, advantage was taken of the recent observations that it is possible to induce the acquisition of metastatic ability in initially nonmetastatic cancer cells by means of DNA transfection (12–15). Using this method, Treiger and Isaacs (14) developed a series of highly metastatic transfectants, all derived from a single clonal, low metastatic ability, parental Dunning AT-2.1 cell line. Initially, AT-2.1 cells were transfected with a plasmid vector containing the v-H-ras gene to determine whether v-H-ras transfection and its p21 product expression would correlate with a change in the metastatic ability. Three of the nine v-H-ras transfectants with documented integration of the v-H-ras oncogene had low p21 protein expression and did not exhibit an increase in metastatic ability, whereas one cell line expressing intermediate levels of p21 increased its metastatic ability threefold and five cell lines with high p21 expression increased their metastatic ability as much as 5- to 10-fold following v-H-ras transfection. We report here the use of our visual grading system of cell motility to characterize the membrane ruffling, pseudopodal extension, and cell translation of these same transfectants, within their initial passage to determine if there are characteristic changes in cell motility that correlate with the increase in metastatic ability exhibited within the series of v-H-ras transfectants.

MATERIALS AND METHODS

Cell Lines. All cell culture lines used in this study have been previously reported and characterized by Treiger and colleagues (14). The metastatic ability of these cell lines (Table 1) was determined as previously described and reported by Treiger et al. (14). The untransfected cell line AT-2.1 was cloned by limiting dilution from the Dunning R3327 AT-2 rat prostatic adenocarcinoma cell line. DNA transfection of the AT-2.1 cell line was performed by the calcium phosphate method as previously described (14). Cell lines neo 3.5 and neo 3.7 received the plasmid pZipNeoSV(X) which transferred resistance to the antibiotic G418 (growth in media containing G418 was used as selection criteria for positive transfection). The remaining cell lines (neo/ras 4.9, 4.7, 4.1, 4.13, 4.4, 4.16, 4.5, 4.2, and 4.8) received the plasmid pZipNeoSV(X) which transferred resistance to G418 and the gene for the viral H-ras p21 protein. Metastatic ability is expressed as low metastatic when
<30% of rats inoculated in the flank with 10 mg of solid tumor had developed distant metastases at the time of autopsy and high metastatic when >50% had developed distant metastases. Documentation of v-h-ras transformation was previously determined by Southern analysis of the DNA, immunoprecipitation of the v-H-ras product (p21), and immunocytochemical localization of p21 of cells in culture (14). Approximately 10^6 cells were inoculated into T-25 plaque-treated plastic tissue culture dishes (Lux Lab-Tek; Miles Scientific, Naperville, IL) and equilibrated in 5% CO_2:95% air at 37°C in 3 ml of Dulbecco’s Modified Eagle’s Media containing 500 µg/ml of the antibiotic G418 (GIBCO, Grand Island, NY) and 250 nM dexamethasone (Sigma Chemical, St. Louis, MO). Cells were plated at low density to facilitate the microscopic imaging of single cells.

Time-Lapse Videomicroscopy. 12 to 24 h after plating, flasks were sealed and transferred to a 37°C heated microscope stage (Zeiss TRZ 3700, Thornwood, NY) for microscopy. Single cells were viewed with a high resolution black and white video camera (Dage MTI 66; Michigan City, IN) at 400× magnification with an inverted microscope (Zeiss IM35) fitted with a 40× Hoffman modulation contrast objective lens (Zeiss). Images were recorded at 15-s intervals for 3 h on ½ VHS video tape with a time-lapse video recorder (Panasonic TL AG-6050; Secaucus, NY). Ten separate time-lapse videomicroscopic films of isolated cells from each of the 12 cell lines were made under similar conditions.

Visual Motility Grading. Three parameters of cell motility [cell membrane ruffling, pseudopodial extension, and cell translation previously described (21)] were visually graded by an observer who had no knowledge of the identity of the cells. Each cell was given a grade for each parameter from 0, completely motionless to 5, large amounts of motility. The grades for each parameter were then analyzed and also summed and divided by three to yield a motility index score. For each cell line 10 cells were filmed and graded. The scores of the 10 cells were averaged to yield a mean subline grade. Comparison of the motility indices for each parameter from the low metastatic cell lines with the mean values for the cell lines was made under similar conditions.

RESULTS

The AT2.1 cell line, a clonal rat prostatic cancer cell line with low metastatic ability (i.e., <30% of rats inoculated s.c. with 10 mg solid tumor develop distant metastases, Table 1) was transfected with either the neomycin resistance gene alone (neo) or in combination with the v-H-ras oncogene (neo/ras). The metastatic ability did not increase in the two cell lines transfected with the neomycin resistance gene alone (neo 3.5 and 3.7 showed no increase) (Table 1). Four cell lines transfected with the neomycin resistance gene and the v-H-ras oncogene (neo/ras 4.1, 4.9, 4.13, and 4.7) showed either little or no increase in metastatic ability, whereas, five lines transfected with neo/ras (neo/ras 4.8, 4.16, 4.2, 4.4, and 4.5) showed marked increases in metastatic ability (Table 1).

Membrane ruffling, defined as high frequency, low amplitude modulation of well-defined regions of the cell membrane, was visually graded and demonstrated an increase in the high metastatic cell lines (Fig. 1A). The mean visual ruffling grades (Table 2) of the five high metastatic cell lines were all higher than those of the seven low metastastic cell lines. Statistical analysis of the visual ruffling scores for the 70 individual cells studied from the low metastastic cell lines and the 50 individual cells from the high metastatic cell lines differed significantly (Mann-Whitney-Wilcoxon, P < 0.001) (Table 2).

Visual grading of pseudopodial extension, defined as low frequency, high amplitude changes in large regions of the cell membrane, demonstrated similar separation of the high and low metastastic cell lines when the mean values for the cell lines were compared (Fig. 1B). The visual pseudopodial extension scores for the individual cells also showed significant separation (P < 0.001).

The mean values for the visual grading of cell translation (Fig. 1C), defined as movement of a cell either in a straight line or in a random manner from one point to another, produced separation of the cell lines on the basis of metastastic ability. Visual translation grading of the individual cells also produced separation of the low from high metastastic cell lines (P < 0.001). The motility index (sum of the three visual motility parameters divided by three) produced similar separation of the high and low metastastic cell lines (P < 0.001).

Separation of the 30 low metastastic cells that had not been transfected with ras (AT-2.1, neo 3.5 and 3.7) was not significantly different from that of the 40 low metastastic cells that were transfected with ras (neo/ras 4.1, 4.9, 4.13, and 4.7) for all visual grading parameters (ruffling, P = 0.35; pseudopodial extension, P = 0.96; translation, P = 0.40; and motility index, P = 0.90).

DISCUSSION

Cancer cell motility has become one of the most active areas of investigation in the study of invasive tumor growth (22). In 1940, George Gey was the first to use time-lapse cinematography to analyze the movements of cancer cells derived from the spontaneous transformation of normal cells in culture (2). In 1966, Wood and associates demonstrated, using a transparent rabbit ear chamber, that normal tissue cells were nonmotile with the exception of leukocytes and macrophages and that V-2 carcinoma cells migrated within the rabbit ear 200 times faster than macrophages (23). Enteline and Coman demonstrated the variability in the motility of cancer cells and suggested that the degree of motility within a tumor might correlate with its invasive ability (24).

The transformation of a stable, nontumorigenic population of cells to an immortal, tumorigenic, and metastatic phenotype either in vitro or in vivo is a complex and poorly understood process. It is well recognized that the acquisition of the metastatic phenotype by a cell may be a multifactorial process involving many activities such as selective growth advantage,
the ability to separate from the primary tumor, cell locomotion, and physical and enzymatic destruction of host tissues (4). Cell motility may play a critical role in these activities, making it an essential, but not sufficient requirement for metastasis (25).

The Dunning R3327 rat prostatic adenocarcinoma tumor model provides several histologically indistinguishable cancers with varying metastatic ability all of which originated from a single spontaneous tumor (26). No histochemical, biochemical, or morphometric analysis of the Dunning prostatic cancer sublines has been capable of predicting their metastatic ability (26). Mohler et al. demonstrated that five of the in vitro Dunning cancers and cultured normal rat prostate epithelial cells could be identified by a reproducible visual grading system of cell motility (20). The visual grading of three types of cell movements, membrane ruffling, the extension or retraction of pseudopods (pseudopodal extension), and cell translation, provided the information necessary to describe the motility of these cancer cells and allowed assessment of their metastatic ability (21). They showed that Dunning prostatic cancers with high metastatic ability demonstrated high amounts of these types of motility. The Dunning tumors lines are all well established and characterized cancers that derived from a single spontaneous rat prostate tumor. The event(s) leading to the expression of high amounts of cell motility and the development of high metastatic ability in these cancers remains obscure. Whether or not the correlation between the increased cell motility and high metastatic ability seen in the Dunning cancers is a selected artifact of continuous passage in culture or developed with the initial acquisition of metastatic ability was directly examined using this Dunning system.

Treiger and Isaacs (14) developed a series of cell lines with which we could test this hypothesis. They transfected the stable, anaplastic, tumorigenic, low metastatic, and low motile AT-2.1 cell line with either the pZipNeoSV(X) plasmid (neo, G418 antibiotic resistance only) or the praspir6 plasmid (neo/ras, G418 resistance plus the v-H-ras oncogene) and developed 11 transfectant cell lines with varying metastatic ability. Six of these transfected cell lines showed no or only small changes in their metastatic ability (neo 3.5, 3.7, and neo/ras 4.1, 4.9, 4.13, and 4.7) whereas five cell lines (neo/ras 4.8, 4.16, 4.2, 4.4, and 4.5) increased their metastatic ability from 5- to 10-fold. This direct perturbation of these cell lines provided us with highly metastatic cell lines that were previously low metastatic lines and low metastatic that had received the same treatment and did not increase their metastatic ability. This allowed us to study the correlation of the motility of these cell lines with their in vivo metastatic ability within the first few passages in culture.

The data presented in this work demonstrate significantly increased membrane ruffling, pseudopodal extension, and cell translation in the v-H-ras-transfected cell lines with high metastatic ability. No difference in motility was noted between the parental, low metastatic AT-2.1 cell line, the pZipNeoSV(X) neo only transfected cell lines, and the low metastatic v-H-ras-transfected cell lines. These findings extend the observations of the previously documented increase in motility of the highly metastatic, established Dunning cell lines (21). These data suggest that the correlation of increased cell motility and high metastatic ability in an animal model of rat prostatic cancer is an early event in the acquisition of metastatic ability (documented within the first few passages) and not a selected artifact of continued passage. While documenting an early increase in cell motility in a tumorigenic, low metastatic (AT-2.1) v-H-ras-transfected cell line with newly acquired metastatic ability, we have not addressed the question as to what motility changes might occur when a population of nontumorigenic, nonmetastatic cells is transformed to an immortalized, possibly tumorigenic cell line in culture. The ability to reproducibly and repeatedly induce the new development of metastatic ability in an initially nonmetastatic tumor cell by means of DNA trans-
fection coupled with a quantitative, computerized method of mathematically evaluating the motility of large numbers of cells (27) will allow a more precise analysis of the relationship between motility changes and metastatic ability.

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