High Invasiveness Associated with Augmentation of Motility in a fos-transferred Highly Metastatic Rat 3Y1 Cell Line

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ABSTRACT

We previously reported that v-fos transfer to a src-transformed rat 3Y1 cell line enhanced lung metastasis. To clarify the mechanism of this enhancement, we compared various biological factors related to metastatic potential between a fos-transferred highly metastatic cell line (fos-SR-3Y1-202) and the control cell line transferred with genetic marker (pSV2-neo) plus pBR322, neo-SR-3Y1-200. Lung arrest, the effect of lung extract on cell growth, or sensitivity to natural killer cells could not explain the higher metastasis of fos-SR-3Y1-202, compared to findings with neo-SR-3Y1-200. The invasiveness, assessed by penetration through a Matrigel-coated filter was about 5 times higher in fos-SR-3Y1-202 than in neo-SR-3Y1-200; high invasiveness in vitro was also observed in a fos-transferred mixed-population cell line (fos-SR-3Y1-200) and fos-transferred highly metastatic clones. Histopathological evidence of an in vitro tumor also showed the high invasiveness of fos-SR-3Y1-202 cells. To elucidate the causes of the increased invasiveness of fos-SR-3Y1-202, attachment of the cells to Matrigel and its components, related to laminin and type IV collagen, type IV collagenase activity, and motility were examined. Attachment of the cells to the substrate coated on Petri dishes or the activity of type IV collagenase did not differ significantly. On the other hand, cell motility, determined by a new method to directly quantify alteration of cell shape continuously, using video image analysis and computer techniques, was higher in vivo in fos-SR-3Y1-202 than in neo-SR-3Y1-200. Thus, the fos-transferred cell line, fos-SR-3Y1-202 has a high invasiveness, in association with augmentation of motility, hence the enhancement in metastatic potential.

INTRODUCTION

Molecular biological approaches to cancer metastasis, in particular with reference to oncogenes, are being used (1, 2). Because metastasis requires the autonomous growth potential of neoplastic cells, the oncogenes, mainly involved in regulation of cell growth, have been given attention.

Metastatic potential is a complex phenotype consisting of multiple biological factors, and we directed attention to the v-fos nuclear oncogene, which apparently regulates the expression of various genes, as a trigger (3). We transferred the v-fos oncogene into a tumorigenic but low metastatic rat clonal cell line, 3Y1-B clone 1-6 (13), with Schmidt-Ruppin D strain Rous sarcoma virus (14). SR-3Y1-2 was used as the recipient of the v-fos gene (4). fos-SR-3Y1-202 is a representative clonal, highly metastatic cell line established by transfer of pFBJ-2 plasmid (15) containing proviral v-fos DNA and pSV2-neo (16) into SR-3Y1-2 (4). fos-SR-3Y1-203, 205, and 202-F1, which are also highly metastatic clonal cell lines (4, 5), were used for in vitro invasion assay. fos-SR-3Y1-202-F1 was a clone established from a lung-metastatic nodules of fos-SR-3Y1-202 (5). fos-SR-3Y1-201, a low metastatic clone immut uinely transcribing exogenously transferred v-fos DNA, was also used for the in vitro invasion assay. neo-SR-3Y1-200 is a G418r cell line of mixed population transformed with pSV2-neo plus pBR322. The numbers of lung nodules formed by the above cell lines (10 cells/rat i.v.) were as follows (5): SR-3Y1-2, 7-13; neo-SR-3Y1-200, 10-48; fos-SR-3Y1-201, 2-3; fos-SR-3Y1-202, 150-234; fos-SR-3Y1-203, 128-150; fos-SR-3Y1-205, 157-208. fos-SR-3Y1-201-F2 produced much the same lung nodules as fos-SR-3Y1-202.

MATERIALS AND METHODS

Cell Culture. All cells were maintained in DMEM* containing 10% fetal bovine serum in a humidified atmosphere of 10% CO2 and 90% air. SR-3Y1-2 was a clonal cell line established by infection of a normal rat fibroblast cell line, 3Y1-B clone 1-6 (13), with Schmidt-Ruppin D strain Rous sarcoma virus (14). SR-3Y1-2 was used as the recipient of the v-fos gene (4). fos-SR-3Y1-202 is a representative clonal, highly metastatic cell line established by transfer of pFBJ-2 plasmid (15) containing proviral v-fos DNA and pSV2-neo (16) into SR-3Y1-2 (4). fos-SR-3Y1-203, 205, and 202-F1, which are also highly metastatic clonal cell lines (4, 5), were used for in vitro invasion assay. fos-SR-3Y1-202-F1 was a clone established from a lung-metastatic nodule of fos-SR-3Y1-202 (5). fos-SR-3Y1-201, a low metastatic clone immut uinely transcribing exogenously transferred v-fos DNA, was also used for the in vitro invasion assay. neo-SR-3Y1-200 is a G418r cell line of mixed population transformed with pSV2-neo plus pBR322. The numbers of lung nodules formed by the above cell lines (10 cells/rat i.v.) were as follows (5): SR-3Y1-2, 7-13; neo-SR-3Y1-200, 10-48; fos-SR-3Y1-201, 2-3; fos-SR-3Y1-202, 150-234; fos-SR-3Y1-203, 128-150; fos-SR-3Y1-205, 157-208. fos-SR-3Y1-201-F2 produced much the same lung nodules as fos-SR-3Y1-202.

Immunostaining. M2 antisemur (17) against M2 peptide having a common sequence for c-fos and v-fos proteins was kindly provided by Dr. I. Verma (The Salk Institute, San Diego, CA). V2 antisemur (17) was prepared by immunizing a rabbit with v-fos-specific V2 peptide, NH2-(Cy5)-Val-Phe-Pro-Glu-Arg-Phe-Pro-Ser-Thr-COOH, conjugated with bovine serum albumin according to the method of Curran et al. (17). For immunostaining, the cells were plated on glass coverslips, cultured for at least 24 h, rinsed with PBS prewarmed at 37°C, and fixed for 20 min with 3% paraformaldehyde in PBS. These fixed cells were then washed with PBS containing 10 mM glycine, then permeabilized for 5 min with 1% Nonidet P-40 in PBS plus glycine, and washed as before. The antibodies against M2 peptide or V2 peptide diluted 1:100 or 1:400, respectively, were poured over the cells and the preparation was incubated for 1 h. After the cells with PBS plus glycine were washed, they were exposed to the second antibody, rhodamine- or fluorescein-conjugated IgG fraction of goat anti-rabbit IgG (Cappel, Cochranville, PA) diluted 1:400 for 30 min. These cells were then observed under an

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4 The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; NK, natural killer cells; BSA, bovine serum albumin; MHC, major histocompatibility complex.

5 Unpublished data.
Fig. 1. Indirect-immunofluorescence staining of fos-SR-3Y1-202 and neo-SR-3Y1-200 with antibodies against V2 peptide or M2 peptide. a, the same field as in c viewed under phase-contrast optics; b, the same field as in d viewed under phase-contrast optics; c, neo-SR-3Y1-200 cells stained with antibody against V2 peptide; d, fos-SR-3Y1-202 cells stained with antibody against V2 peptide; e, neo-SR-3Y1-200 cells stained with antibody against M2 peptide; f, fos-SR-3Y1-202 cells stained with antibody against M2 peptide. × 40.

Olympus fluorescence microscope (Olympus, Tokyo, Japan).

Measurement of Tumor Cell Arrest in the Lung. Assay of lung arrest was done according to the method of Fidler (18). Each rat was given i.v. 2 × 10^7 of [%^131]iododeoxyuridine-labeled cells. Twenty min or 3, 6, or 18 h after the inoculation, the lungs were removed, briefly washed with tap water, dried using filter paper, and placed overnight in vials containing 70% ethanol. Three animals were used for each point. The radioactivity retained in the lung was measured in a gamma counter (Aloka; ARC-361).

Effect of Lung Extract on Cell Growth in Vitro. Preparation of lung extract and assay of growth-stimulating activity were done according to the method of Yamori et al. (19). Cells (5 × 10^5) were suspended in DMEM containing 1% fetal bovine serum and plated into a 60-mm Petri dish. After incubation for 24 h at 37°C, the attached cells were counted (time 0 count) and fed DMEM containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer and 100 µg of protein of the lung extract per ml prepared from fresh lungs of F344 rats. Growth index was defined by dividing the cell counts at each time point by the time 0 counts.

Assay for NK Activity. NK activity was assayed according to the method of Kawano et al. (20). Target cells labeled with Na_2[^35]CrO_4 were mixed with effector cells at ratios of 100:1 and 50:1, in quadruplicate, and incubated for 4 h at 37°C in 10% CO_2 and 90% air. After centrifugation, the radioactivity in the supernatants was measured in a gamma counter.

\[
\% \text{ of cytotoxicity} = \frac{\text{Test cpm} - \text{spontaneous cpm}}{\text{Total cpm} - \text{spontaneous cpm}} \times 100
\]

Spontaneous cpm was determined with the supernatant from samples of target cells incubated without effector cells, and the total release was determined from those incubated with 10% Triton X-100.

Invasion Assay. In vitro invasiveness was assessed using the method of Albini et al. (21) and basement membrane Matrigel, an extract of the Englebreth-Holm-Swarm tumor, prepared according to the method of Kleinmann et al. (22). Polycarbonate filters, 8 µm pore size (Nuclepore) were coated with 50 µl (1 mg of protein/ml) of basement membrane Matrigel and dried under a hood. The coated filters were then placed in Boyden chambers, and cells (2.6 × 10^6) suspended in DMEM containing 0.1% BSA were applied to the upper chamber. The lower chamber was filled with conditioned medium obtained by incubating NIH-3T3 cells for 24 h in DMEM containing 0.005% vitamin C and 0.1% BSA. Cells migrating to the lower surface of the filters were fixed, stained, and counted. The number of cells which penetrated a filter was counted in 10 fields under a 200-fold magnification. Assays were carried out in triplicate at 37°C in 10% CO_2 and 90% air for 5 h.

Cell Attachment to Substrate-coated Bacterial Dishes. Tumor cells (2 × 10^5) were suspended with 1 ml of serum-free DMEM containing 1% BSA, and plated in triplicate in 35-mm Petri dishes, either uncoated or coated with 50 µg of Matrigel, human type IV collagen, or mouse laminin.

At 15-min intervals for Matrigel or 30-min intervals for type IV collagen and laminin, the cultures were washed gently with PBS and the attached cells were trypsinized. The number of cells was counted using a hemocyte calculator and/or Coulter counter.

Histopathological Examination. Tumors formed by inoculation of 1 × 10^6 cells into the thigh of F344 rats were dissected, fixed with 10% formalin, and stained with hematoxylin and eosin. For one cell line, three tumors were used for morphological examination.

Type IV Collagenase. Activity of type IV collagenase was assessed according to the method of Nakajima et al. (23). N-[propionate-2,3,4-3H]-propionylated human type IV collagen (Dupont, Boston, MA) was determined using a liquid scintillation counter and expressed as counts per minute (cpm) of collagenase activity.
Fig. 2. Comparison of several biological phenotypes relating to metastatic potential between neo-SR-3Y1-200 and fos-SR-3Y1-202. (A) pulmonary retention of i.v. injected neo-SR-3Y1-200 and fos-SR-3Y1-202. Cell suspension of 0.5 ml containing 1 x 10^6 viable cells (about 7 x 10^6 cpm) were given through the tail vein of rats. Three animals were used for each point. O, neo-SR-3Y1-200; ○, fos-SR-3Y1-202. P > 0.1 (not significant at any point between the cells, evaluated by Student's t test). B, in vitro growth in the medium containing lung extract. Growth medium was replaced on Day 0 with serum-free DMEM, with or without 100 µg protein of lung extract per ml. Growth index was determined, as described in "Materials and Methods." Three dishes were used for each point. O, neo-SR-3Y1-200 with lung extract; ●, fos-SR-3Y1-202 with lung extract; ●, neo-SR-3Y1-200 without lung extract; ▼, fos-SR-3Y1-202 without lung extract. P > 0.1 (not significant at any point between the cells, evaluated by Student's t test). C, sensitivity of fos-transferred cells to NK. "Cr-labeled target cells were incubated with rat spleen cells for 4 h. The "Cr released in the supernatants was counted. The effector:target ratio was 100:1 and 50:1. The percentage of cytotoxicity was determined as described in "Materials and Methods." Spleen donor animals were given 0.6 mg polyinosinate-polycytidylate in 1.0 ml of PBS i.v. on 24 h before the assay. ●, fos-SR-3Y1-202; ▲, neo-SR-3Y1-200. P < 0.05 (significant at both effector:target ratios, evaluated by Student's t test).

mixed with nonlabeled human type IV collagen and adjusted at 100,000 cpm/200 µg/ml in 0.5 M acetic acid. Aliquots (3,000 cpm) of the type IV collagen solution were placed in each well of 96-multiwell tissue culture plates and dried overnight in an air flow hood. Cells (1 x 10^4) per 200 µl in DMEM containing 10% fetal bovine serum or trypsinized culture supernatant prepared by the method of Nakajima et al. (23) were placed on a dried 1H-collagen film and incubated at 37°C in a humidified atmosphere (10% CO_2-90% air). At various intervals, the culture supernatant was removed and mixed with 50 µl of ice-cold 10% trichloroacetic acid and 0.5% tannic acid, in a sample tube. After 30 min incubation at 4°C, the mixture was centrifuged at 10,000 x g for 10 min at 4°C to precipitate the undigested materials. The radioactivity of the supernatant was measured in a liquid scintillation counter.

Cell Motility. To measure cell motility, 10^5 cells of neo-SR-3Y1-200 and fos-SR-3Y1-202 were plated on a regular Petri dish with regular culture medium containing 10% fetal bovine serum. Two days later, the cells were used for measurement of cell motility. Cell behavior was recorded by a video recorder for a few min. Cell motility was directly quantitated by measuring total brightness in a window on the TV monitor, as a result of the accumulation of trace images reflecting changes in cell morphology (24). Trace images were obtained by subtracting a digital image for cells in any one video frame from a digital image for the same cells in the subsequent frame (600 video frame intervals, 20-s intervals). The trace images for the particular cells were accumulated four times. With the system, the Allen video-enhanced contrast-differential interference contrast microscopy was used to calculate the absolute values of brightness of the trace image corresponding to cell motility. The qualitative value of cell motility was defined as the motility index (MI), using the formula

\[ MI = \frac{(B_t - B_{t-4})}{N/t} \]
Expression of fos Protein. Expression of fos protein was confirmed by staining with antibody against the V2 peptide of fos protein. As shown in Fig. 1, nuclei of a fos-transferred highly metastatic cell line, fos-SR-3Y1-202, stained more strongly than the control cell line, neo-SR-3Y1-200 with the antibody against V2 peptide. This would suggest that fos proteins are produced from exogenously introduced v-fos DNA. In some cases the cytoplasm was stained. It remains to be determined whether this minor staining is due to cytoplasmic distribution of v-fos and/or fos-related proteins. Selective staining of nuclei of fos-SR-3Y1-202 was also seen using the antibody against M2 peptide (Fig. 1, e, f).

Factors Not Explaining High Metastasis of fos-SR-3Y1-202. Because the experimental metastasis of fos-SR-3Y1-202 was higher than that of neo-SR-3Y1-200 (5), we first examined whether lung arrest would differ between them. When 125I-dUrd-labeled cells were injected i.v. into F344 rats and monitored by counting the released radioactivity of H-labeled pyroninted collagenase (25 units/ml), effectively digested type IV collagen in this system. The counts of radioactivity were not significantly different (P > 0.05) at any time point in the cell suspension or in the trypsin-treated one, between fos-SR-3Y1-202 and neo-SR-3Y1-200.

One other factor affecting the invasiveness is cellular motility. Using a video-imaging analysis, we directly measured motility of the cells plated on regular Petri dishes. Fig. 6 shows the phase-contrast microscopic image of the cells (left), the Allen video-enhanced contrast-differential interference contrast microscopic image (middle), and the cumulative trace image (right). The bright region of the right picture corresponds to accumulated traces. This trace image clearly shows that the motility of fos-SR-3Y1-202 was much larger than that of neo-SR-3Y1-200, as shown in Fig. 6a. One other factor affecting the invasiveness is cellular motility.

DISCUSSION

We obtained evidence that the v-fos-transferred highly metastatic cell line, fos-SR-3Y1-202, is more invasive than the control cell line transferred with pSV2-neo plus pBR322, neo-SR-3Y1-200, and that the augmented invasiveness was associated with increase in cell motility. This augmentation of motility would explain the high invasiveness of fos-SR-3Y1-202. Since other biological factors relating to metastasis, including lung arrest, growth potential, or sensitivity to NK, could not explain the high metastasis of fos-SR-3Y1-202, the high invasiveness appeared to be the main factor responsible for the high potential in experimental and spontaneous metastasis of fos-
SR-3Y1-202. Augmentation of invasiveness was also seen in a fos-transferred mixed-population cell line, fos-SR-3Y1-200, and the fos-transferred highly metastatic clones (fos-SR-3Y1-203, 205, and fos-SR-3Y1-202-F1); thus, v-fos transfer generally induces a high invasiveness as well as high metastasis in SR-3Y1-2.

Kerbel et al. (26) and Van Roy et al. (27) stated that metastatic potential is sometimes affected by the gene transfer procedure itself or by the introduced genetic marker pSV2-neo alone. Therefore, we repeated independent gene transfer experiments, with the v-fos gene, using SR-3Y1-2 as the recipient. Transfer of the v-fos gene reproducibly increased the metastatic potential (28).

Liotta proposed a three-step hypothesis describing the sequence of biochemical processes that take place during tumor cell invasion of the extracellular matrix: matrix attachment, matrix dissolution, and tumor cell locomotion (29). We found that attachment to Matrigel and its components, such as type IV collagen and laminin, did not significantly differ between low and highly metastatic cell lines or IV collagenase activity. Cell motility was, however, augmented in a highly metastatic cell line, as compared with the control cell line. Thus, the increase in invasiveness in the fos-transferred highly metastatic cell line seems to be linked to augmentation of cell motility, although unknown lytic enzymes may be involved.

The augmented motility in highly metastatic cell lines was noted by other investigators (30–32). The motility measured in the present study mainly reflects ruffling, pseudopodial extension, and partial cell translation, because of the short period of measurement. In particular, increase in pseudopodal extension was observed by electron microscopy. Increases in ruffling and pseudopodal extension probably lead to enhancement of directed cell locomotion, an event important for invasion. Recently, Partin et al. (33) noted that Fourier motility coefficients measuring pseudopodal extension correlated best with metastatic potential, as compared with other parameters, including cell translation. The molecular mechanisms involved in alteration of cellular motility appear to include changes in expression of cytoskeletal proteins and/or secretion of autocrine motility factors. In B16 melanoma cell lines, we observed an alteration

of a new type of actin coexpressed with β- and γ-actin accompanying increases in invasiveness and metastasis (34–36). By comparing the expression of cytoskeletal proteins between fos-SR-3Y1-202 and neo-SR-3Y1-200, we also noted alteration in expression of actin-associating proteins accompanying increases in the metastatic potential (37).

The positive correlation between fos expression and metastatic potential was noted by Yughi et al. (38) in the spontaneous rat mammary carcinoma. The biological factors responsible for the metastatic potential were not determined. A correlation between expression of MHC class I and expression of the fos gene was noted by Kushtai et al. (39). They reported that the c-fos oncogene and H-2K are expressed in low-metastatic Lewis lung carcinoma clones, but not in high-metastatic clones. We observed the relatively high sensitivity of fos-SR-3Y1-202 to NK, as compared with neo-SR-3Y1-200 (Fig. 2C). This may relate to alteration in the expression of MHC class I-like antigens in the recipient rat cells, consistent with Kushtai’s observation. In our system, however, the increase in invasiveness by transfer with the fos oncogene appears to surpass the increase in the sensitivity to NK, resulting in the enhancement of metastatic potential, while this is not the case in Kushtai’s system. On the other hand, when we transferred the v-fos gene into a ras-transformed rat 3Y1 cell line, the sensitivity to NK of the recipient cells tended to decrease and there was an increase in lung metastasis.6 Alon et al. showed that transfection of the H2-D3′ gene into nonmetastatic T-10 fibrosarcoma lines increased the metastatic potential (40). Thus, the metastatic potential and/or sensitivity to host defense systems apparently depend on which type of MHC class I-like antigens are expressed, and alteration in the expression of antigen and other genes by the fos gene may depend on the cell type.

Transient expression of c-fos by growth factors, tumor promoter, calcium, and differentiation-inducing factors is considered to trigger the expression or suppression of some gene sets, resulting in the promotion of growth or differentiation (41, 42). These phenomena also indicate that expression of the fos gene does not always result in the same biological consequences. Although it is well documented that the active ras oncogene induces malignant transformation in NIH 3T3 cells and other fibroblast cells, Noda et al. (43) noted that v-Ki-ras and v-Ha-ras induces differentiation-associated properties in the rat neoplastic cell line, PC12. The cell dependency of biological consequences by gene transfer was also noted in studies on metastasis. Transfer of the active ras oncogene into NIH 3T3 cells or rat fibroblasts induces or increases the metastatic potential (44) but does not do so in C127 mouse mammary carcinoma (45). Thus, the biological effects of a certain gene product appear to depend on the state or the type of recipient cells.

The present biological analysis suggested that the v-fos product affects the expression of genes related to invasiveness in the SR-3Y1-2 cell line, probably relating to cytoskeletal proteins, autocrine motility factors, and lytic enzymes (46, 47). Molecular biological investigations to identify the fos-regulated genes, with reference to metastasis, are under way, with particular attention directed to transcriptional transactivation activity of the fos product.

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