Independent Regulation of Invasion and Anchorage-independent Growth by Different Autophosphorylation Sites of the Macrophage Colony-stimulating Factor 1 Receptor

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ABSTRACT

Invasion of tissue by macrophages and implantation into the uterine wall by placentantrophoblasts are known to be regulated by the macrophage colony-stimulating factor (CSF-1) and its receptor (CSF-1R, the product of the c-fms proto-oncogene). Recently, the clinical importance of CSF-1 and CSF-1R in invasive breast carcinoma has been recognized, but the significance of coexpression of CSF-1 and CSF-1R in mammary epithelial cell invasion has not been explored. In the present study, we investigated the invasive potential of a noninvasive, CSF-1R-negative, mouse mammary epithelial cell line (HC11) expressing a high level of CSF-1, which was stably transfected with the mouse wild-type CSF-1R. Compared with parental cells, transfected cells expressing a wild-type CSF-1R invaded 100-fold more efficiently through a barrier of reconstituted basement membrane (Matrigel) and formed colonies in soft agar, whereas the cellular growth rate was only slightly increased. Analysis of cell-conditioned medium by zymography and quantitative enzyme activity assays showed that clones transfected with a wild-type CSF-1R expressed significantly higher levels of urokinase-type plasminogen activator than did untransfected clones. Furthermore, after injection into the tail veins of BALB/c mice, CSF-1R-expressing clones also produced a 10-fold higher incidence of lung tumors than the parental cell line. We also analyzed HC11 clones transfected with CSF-1R mutated at two major autophosphorylation sites (Tyr → Phe807 and Tyr → Phe721). Mutation at Tyr807 eradicated the stimulatory effect of Fms on the invasive ability of HC11 cells and substantially reduced the metastatic potential of the transfected clones but did not alter the Fms-induced anchorage-independent growth in soft agar. In contrast, mutation at Tyr721 of Fms had no effect on invasion as measured in the in vitro assay but markedly abolished Fms-induced colony formation in soft agar and eradicated the metastatic potential of the transfected clones. Our results suggest that expression of CSF-1R can facilitate cellular invasion and anchorage-independent growth in mammary epithelial cells, and these two processes are independently regulated by separate phosphotyrosine sites of CSF-1R.

INTRODUCTION

The proto-oncogene c-fms and its viral oncogene derivative v-fms (the transforming gene of the McDonough feline sarcoma virus) encode a growth factor receptor with an intracellular tyrosine kinase domain, the ligand of which is CSF-1 (1—3). The expression of c-fms was originally described in cells of the monocyte-macrophage lineage in which the physiological functions of CSF-1 and its receptor during monocytic differentiation and macrophage activation are reasonably well understood (4, 5). Normal placental trophoblast epithelium expresses high levels of CSF-1R, and activation of trophoblastic CSF-1R by the locally high levels of CSF-1 produced by the endometrial epithelium is essential for normal embryonic implantation and placentant development (6, 7).

Recently, the clinical importance of the expression of CSF-1 and its receptor by malignant epithelial cells of breast, ovarian, and endometrial carcinomas has been recognized (8—13). We and, more recently, other groups have demonstrated that overexpression of CSF-1R in ovarian and endometrial tumor epithelium is strongly associated with and implicated in the development of clinically aggressive malignant neoplasms (13, 14). At least 90% of invasive breast carcinomas have been shown to express high levels of CSF-1R (9). Also, isolated, stromally invasive breast carcinoma cells consistently expressed CSF-1, which was often not expressed in adjacent noninvasive, in situ carcinoma (15).

These observations raised the intriguing possibility that a hematopoietic and placental cell growth and differentiation factor, CSF-1, and its receptor might render malignant epithelial cells invasive, much as they promote macrophage differentiation and migration (4) and stimulate placental invasion into the uterine wall (6). Several in vitro studies already have provided some evidence of a stimulatory effect of the expression of CSF-1R on invasion in tumor cell lines derived from metastatic carcinoma of the breast, lung, and ovary (16, 17). The question of whether CSF-1 and its receptor could initiate an invasive phenotype in nonneoplastic cells has not been addressed.

In the present study, we investigated the invasive phenotype of a normal, noninvasive mouse mammary cell line (HC11), which expresses CSF-1 but not its receptor, before and after stable transfection either with the wild-type mouse c-fms gene or with c-fms genes mutated at the codon for either of two major autophosphorylation sites (Tyr → Phe807 or Tyr → Phe721). Previous analysis of such phosphorylation site mutants showed that Tyr807 and Tyr721 play important roles in coupling the CSF-1R to intracellular signal transduction pathways (18, 19). The Tyr807 site in particular was demonstrated to be crucial for CSF-1R-dependent monocytic differentiation (18), whereas the Tyr721 site of the CSF-1R is required for CSF-1-dependent mitogenesis (20). By expressing CSF-1R mutants with Tyr → Phe amino acid substitutions at positions 807 and 721 in HC11 cells, we were also able to study the importance of specific tyrosine autophosphorylation sites of CSF-1R in the autocrine activation of the CSF-1R by its ligand in normal mammary epithelial cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture Methods. The mouse fibroblast cell line NIH3T3 was obtained from the American Type Culture Collection (Rockville, MD). The NIH3T3-FMS cell line (also known as p2EN-FMS), which expresses very high levels of murine CSF-1R was obtained from Rohrschneider et al. (21). HC11 cells were originally isolated from mammary glands of midpregnant BALB/c mice by Hynes et al. (22). The CSF-1-dependent macrophage cell line 2FS (also known as BAC1.2FS) was cloned by Morgan et al. (23).
The wild-type murine c-fins eDNA clone was excised from the pMZen (c-fins) plasmid, then electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde, then transferred to a nylon membrane (GeneScreen; DuPont). Filters were hybridized overnight at 42°C in the presence of 10% dextran sulfate, 50% formamide, 1X-50, 0.1% SDS, and 0.1 mg/ml salmon sperm DNA, and then washed. RNA Extraction and Northern Blot Analysis. Total cellular RNA was isolated by the guanidinium-cesium chloride method (26). RNA samples were electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde, then transferred to a nylon membrane (GeneScreen; DuPont). Filters were hybridized overnight at 42°C in the presence of 10% dextran sulfate, 50% formamide, 1 M NaCl, 1% SDS, 100 µg/ml salmon sperm DNA, and a 32P-labeled murine c-fms probe (0.6-kb EcoRI-KpnI fragment of pZen113X c-fms plasmid; Ref. 21). After autoradiography, filters were stripped and rehybridized successively with a murine CSF-1 cDNA probe (2.0-kb EcoRI fragment of CSF53 plasmid; Ref. 28) and with a human GADPH cDNA probe (0.8-kb PstI-Xhol fragment of HHCH32; ATCC 8105). Specific hybridization was visualized by Kodak XAR-5 film autoradiography. Lysate Preparation, Immunoprecipitation, PAGE, and Western Blot Analysis. Cells were washed twice with ice-cold PBS plus 0.5 mM sodium orthovanadate and kept on ice throughout lysate preparation. Cells were lysed in ice-cold radioimmunoprecipitation assay buffer (10 mM sodium phosphate buffer (pH 7.2), 150 mM NaCl, 1 mM EDTA, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, and freshly added 22 µM aprotinin and 1 mM sodium orthovanadate), scraped from the culture vessel surface, and vortexed and left on ice for 10 min. Debris was removed by centrifugation (12,000 × g) for 5 min at 4°C. Protein concentrations were measured using the Bio-Rad protein assay. For immunoprecipitation, lysates containing 1 mg of protein were added to 50 µl (packed volume) of washed protein A-agarose beads plus antibody at 1:125 (anti-fms, 06-175, an affinity-purified polyclonal antibody against the cytoplasmic domain of mouse CSF-1R; Upstate Biotechnology, Inc.). The samples were tumbled at 4°C for 2 h, centrifuged to pellet the beads, and washed first with cold radioimmunoprecipitation assay buffer and then with PBS plus 0.5 mM sodium orthovanadate. The beads were resuspended in 25 µl of 2X sample buffer and boiled. After centrifuging for 20 s (12,000 × g) for 5 min, the supernatant was used for SDS-PAGE immediately. SDS-PAGE and Western blotting with the anti-fms polyclonal antibody (Upstate Biotechnology) were carried out as described previously (29).

DNA Transfection. For transfection, the Lipofectamine reagent (Life Technologies) was used as described. Both Lipofectamine and 3 µg of plasmid DNA were diluted separately into 100-µl aliquots of Opti-MEM I (Life Technologies). Combined aliquots were incubated at room temperature for 45 min to allow the formation of DNA-lipid complexes. The complexes were then diluted with 1.8 ml of Opti-MEM I medium, and the mixture was added to subconfluent (~105) cells in 60-mm Petri dishes. Cells were exposed to complexes for 24 h under standard culture conditions, after which the medium was replaced with fresh culture medium. After further incubation for 48 h, the medium was replaced with selection medium containing gentamicin sulfate (300 µg/ml). Gentamicin sulfate-resistant clones were detected approximately 18 days after transfection, and the selected clones were expanded in the presence of 300 µg/ml gentamicin sulfate.

Chromogenic Assays of uPA. To determine uPA production, 2 × 105 cells were seeded in 60-mm Petri dishes coated with basement membrane matrix (Matrigel, 1 µg/mm2; Collaborative Research) and incubated for 24 h. The medium was then changed to DME/F12 medium (Sigma) containing 0.5 mg/ml BSA (Sigma). After 24 h, the conditioned medium was collected and concentrated using Centricon 10 concentrators (Amicon). Cells were harvested using standard trypsinization procedures and counted by hemacytometer for normalization of results to cell number. A chromogenic microtiter plate assay was used to measure uPA activities in the conditioned medium as described previously (30). Briefly, incubations were carried out in 150-µl volumes in 96-well microtiter plates at 37°C for 4 h. Incubation volumes consisted of 50 µl of sample or uPA standards (high molecular weight urokinase; American Diagnostica), 50 µl of buffer (50 mM Tris with 0.1% polyethylene glycol 8000, 6 mM 6-aminohexanoic acid (Sigma), and 0.1 mg/ml bovine plasma (American Diagnostica)), and 50 µl of 0.4 mM chromogenic plasmid substrate NASA (American Diagnostica). When indicated, the bovine plasmid was omitted from the buffer to serve as a control for plasminogen-independent hydrolysis of NASA. Hydrolysis of the colorless NASA by plasmin results in production of the yellow p-nitroanilide; production of p-nitroanilide was quantitated by measuring absorbance periodically at 405 nm using a Microplate reader (Dynatech Laboratories, Inc.).

Cloning cDNAs Encoding the Wild-Type or Mutated Mouse CSF-1R. The wild-type murine c-fms cDNA clone was excised from the pMZen (c-fms) retrovirus vector (21). The 5.1-kb EcoRI-Ndel fragment containing the entire coding region of c-fms gene was inserted initially into the EcoRI-Ndel polylinker sites of the pPTBlue(R) vector to facilitate the further cloning of the other constructs. Subsequently, the c-fms fragment was excised from the pPTBlue(R) vector using EcoRI-XbaI restriction enzymes and subcloned into the respective sites of the pDNA3 expression vector (Invitrogen). Single-point mutations (Tyr —> Phe and Tyr —> Phe) were introduced into murine c-fms and cloned into the pZen113 plasmid by Carberg and Rohrschneider (25). The 3.1-kb BamHI fragment from each mutant was excised and subcloned into the BamHI polylinker site of the pcDNA3 expression plasmid. The correct orientations and sequences of all above constructs were verified by restriction mapping and direct sequence analysis (26). Antibodies and Protease Inhibitors. The murine mAb to the B chain of human uPA (moAb 394), which recognizes all forms of human uPA, including both receptor-bound uPA and the high molecular weight form of the human uPA protein (27), was purchased from American Diagnostica. Murine IgG (Sigma) was used as a control antibody in the invasion assay. The human recombinant metalloproteinase inhibitors TIMP1 and TIMP2 were obtained from Oncogene Science.

Zymographic Assay of uPA and Collagenase. Qualitative zymographic analysis of uPA and collagenase was performed as described previously (31, 32). Briefly, uPA zymogram gels consisted of 1% SDS and 7.5% polyacrylamide impregnated with casein at 0.08% (substrate for plasmin) and with plasminogen at 25 ng/ml (substrate for plasminogen activator). Collagenase zymogram gels contained 1% SDS and 10% polyacrylamide impregnated with 1 mg/ml collagen. After electrophoresis, the gels were washed twice for 30 min each in 2.5% Triton X-100 solution at 4°C to remove the SDS from the gels. The gels were then incubated for 16 h at 37°C in substrate buffer (50 mM Tris-HCl, 15 mM EDTA (pH 8.0) for casein-plasminogen gels and 50 mM Tris-HCl, 5 mM CaCl2 (pH 8.0) for collagen gels) to allow proteolysis. The gels were then stained with Coomassie blue (G250; Bio-Rad) for 1 h and destained in 50% methanol and 10% acetic acid for 4 h. Proteinase activity was visualized as clear bands, indicating proteolysis of the substrate protein. Photographs of SDS-substrate gels are presented as negative images.
For sample preparation, conditioned, serum-free media were collected from cultures containing equal numbers of cells (10⁶), diluted with 2X sample buffer without mercaptoethanol [65 mM Tris (pH 6.8), 1% SDS, 10% glycerol, and 0.006% bromophenol blue] and loaded immediately on zymogram gels.

**Invasion Assay.** Cell invasiveness was determined using an invasion chamber system (Collaborative Research; Ref. 33) of cell culture inserts (6.5-mm diameter) with 8-μm pore membranes placed in 24-well culture plates. Prior to the assay, the inserts were coated with basement membrane matrix (Matrigel, 1 μg/mm²; Collaborative Research), dried for 4 h in a laminar flow hood, and rehydrated by pipetting DME/F12 medium (Sigma) into the inserts. Cells (5 × 10⁵) suspended in DME/F12 medium (Sigma) containing 0.1% BSA were then seeded into the upper chamber in 200 μl of medium, and DME/F12 medium (Sigma) supplemented with 10% NuSerum was placed into the lower chamber. Cells were incubated at 37°C in 5% CO₂ for 72 h. At the end of the incubation, the cells on the upper surface of the membrane were completely removed by wiping with a cotton swab. The membranes were fixed in methanol and were stained with H&E. The invasive cells adhering to the lower surface of the membrane were counted under a light microscope. When indicated, urokinase mAb 394 (50–150 μg/ml; American Diagnostica), metalloproteinase inhibitors TIMP1 or TIMP2 (3 μg/ml each; Oncogene Science), or nonspecific murine IgG (Sigma) were added to the cells 30 min before the assay. Each invasion assay was performed a minimum of six times. The cells used in the invasion assay were cultured in the presence of 10% NuSerum, rather than 10% fetal bovine serum or 10% calf serum, to reduce the presence of protease inhibitors.

**Experimental Metastasis.** The ability of HC11 cells to form lung tumors was assayed as detailed previously (34). Subconfluent cultures of HC11 clonal cells were lightly trypsinized (0.125% trypsin; Life Technologies), washed in PBS, spun down, and resuspended in DME/F12 medium (Sigma) supplemented with 10% fetal bovine serum and 1 mg/ml of BSA. Viable cells were counted using trypan blue exclusion and were adjusted to the appropriate concentration (10⁵, 10⁶, or 10⁷ cells/ml). Cells were injected in a total volume of 0.2 ml into the lateral tail veins of 2.5-month-old BALB/c-nu mice. The mice were sacrificed by cervical dislocation 50 days after injection; lungs were removed, fixed in Bouin’s solution overnight, and stored in 70% alcohol. Tumor nodules on the lung surfaces were counted using a dissecting microscope. Groups of mice injected with 2 × 10⁵ and 2 × 10⁶ cells had adequate numbers of nodules for rigorous analysis and were used to assess the difference between the cell lines.

**RESULTS**

**Establishment of HC11 Clonal Cells Expressing Significant Levels of the Wild-Type or a Mutated (Tyr → Phe⁸⁰⁷ and Tyr → Phe⁷²¹) Mouse CSF-1R.** A 3.1-kb fragment of the wild-type murine c-fms gene containing the entire coding region with minimal untranslating flanking sequences (21) and two point-mutated (Tyr → Phe⁸⁰⁷ and Tyr → Phe⁷²¹) copies of this fragment were independently inserted into the pcDNA3 eukaryotic expression vector (see “Materials and Methods”). This expression vector incorporates the promoter sequence of the immediate early gene of human cytomegalovirus to drive efficient, constitutive transcription in cells of epithelial origin and expresses a neomycin resistance gene, which serves as a selectable marker. The wild-type c-fms construct and the two different mutated c-fms constructs were stably transduced into an individual subclone of HC11 mouse mammary epithelial cells, and colonies that grew out in the presence of gentamicin sulfate were selected. Individual clones first were screened for expression of c-fms mRNA by Northern analysis (Fig. 1). Although no detectable c-fms transcript levels were observed in RNAs isolated either from the untransfected original cell line (HC11; Fig. 1, Lane 1) or from the NIH3T3 mouse fibroblast cell line (negative control for the expression of c-fms mRNA; Fig. 1, Lane 5), HC11 clones transfected with c-fms expressed significant levels of the 3.1-kb c-fms transcript (Fig. 1, Lanes 2–4).

**CSF-1 transcript levels were also assayed by Northern blot analysis in the untransfected and c-fms-transfected HC11 clonal cells. Because NIH3T3 cells express readily detectable levels of the CSF-1 transcript (21), we included NIH3T3 cells as a positive control for CSF-1 expression on the same Northern blot. Fig. 1 demonstrates that the dominant 4-kb and the smaller 2.3-kb transcripts of CSF-1 (6) were found to be expressed at readily detectable levels in all cell lines studied. However, in HC11 clones expressing the c-fms transcript mutated at Tyr⁸⁰⁷, we observed a striking induction of the CSF-1 transcript level. We are currently designing experiments to further investigate the potential regulatory effect of the Tyr⁸⁰⁷ site of Fms on the expression of the CSF-1 gene.

**Stimulation of Invasion and Clonogenic Growth by CSF-1R.** Establishment of HC11 Clonal Cells Expressed Significant Levels of the Wild-Type or a Mutated (Tyr → Phe⁸⁰⁷ and Tyr → Phe⁷²¹) Mouse CSF-1R. A 3.1-kb fragment of the wild-type murine c-fms gene containing the entire coding region with minimal untranslated flanking sequences (21) and two point-mutated (Tyr → Phe⁸⁰⁷ and Tyr → Phe⁷²¹) copies of this fragment were independently inserted into the pcDNA3 eukaryotic expression vector (see “Materials and Methods”). This expression vector incorporates the promoter sequence of the immediate early gene of human cytomegalovirus to drive efficient, constitutive transcription in cells of epithelial origin and expresses a neomycin resistance gene, which serves as a selectable marker. The wild-type c-fms construct and the two different mutated c-fms constructs were stably transduced into an individual subclone of HC11 mouse mammary epithelial cells, and colonies that grew out in the presence of gentamicin sulfate were selected. Individual clones first were screened for expression of c-fms mRNA by Northern analysis (Fig. 1). Although no detectable c-fms transcript levels were observed in RNAs isolated either from the untransfected original cell line (HC11; Fig. 1, Lane 1) or from the NIH3T3 mouse fibroblast cell line (negative control for the expression of c-fms mRNA; Fig. 1, Lane 5), HC11 clones transfected with c-fms expressed significant levels of the 3.1-kb c-fms transcript (Fig. 1, Lanes 2–4).

**Effect of Fms Expression on the Growth Rate and Anchorage-independent Growth of Normal Epithelial Cells.** A standard proliferation assay (24) was used to measure the growth of untransfected and c-fms-transfected clonal cells plated on uncoated tissue culture wells. When compared with parental HC11 cells, stable transfectants expressing either the wild-type or one of the two mutated CSF-1Rs proliferated at only a slightly higher rate (less than a 2-fold difference in final cell number at 72 h) at 1 and 10% serum concentrations with either NuSerum or fetal bovine serum supplementation (Fig. 3). Furthermore, precoating the dishes with a basement membrane matrix, Matrigel, did not influence the proliferation of any of the HC11 clones studied (data not shown).

Anchorage-independent growth is a cellular phenotype closely associated with tumorigenicity. The HC11 clones expressing the wild-type or mutated Fms were tested for anchorage-independent growth in...
Fig. 2. Expression of CSF-1R (Fms) by HC11 clones transfected with either the wild-type or mutated c-fms gene. Equal amounts of protein prepared from the different HC11 clones were electrophoretically separated in SDS-polyacrylamide gels and electroblotted to a protein transfer membrane. Bound proteins were incubated with rabbit antimouse Fms antisemum followed by horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, and immunoreactive proteins were visualized by the enhanced chemiluminescence detection system. The transfectants (A, Lanes 2 and 3, and B, Lanes 1 and 2) express detectable levels of CSF-1R-immunoreactive polypeptides with a size consistent with that reported previously for the mature fully glycosylated form (~160 kilodaltons (kDa)) of CSF-1R. Positive control cell lysates were obtained from the NIH3T3-FMS mouse fibroblast cell line (A, Lane 4, and B, Lane 3), which expresses high levels of the mouse CSF-1R protein as a result of the stable integration of an Fms expression vector.

Soft agar supplemented with 0 or 150 µg/ml Matrigel. The qualitative results in Fig. 4 demonstrate the colony sizes obtained after growth of untransfected and c-fms-transfected cells in the presence and absence of Matrigel. Table 1 presents the quantitative data on the colony numbers. Untransfected HC11 cells and HC11 cells expressing Fms mutated at Tyr721 grew poorly in soft agar (either in the presence or absence of Matrigel); clones that did grow formed very small colonies, with fewer than 10 cells (Fig. 4). HC11 cells expressing wild-type or Tyr807-mutated Fms grew readily in soft agar and formed large colonies. Addition of the basement membrane matrix Matrigel caused an increase in both the size and number of the colonies (Fig. 4B and Table 1). The addition of exogenous CSF-1, however, had no effect on the size or number of colonies for any of the cell lines studied (data not shown).

Invasive Ability of HC11 Cells Expressing Fms. The invasive potentials of the untransfected and Fms-transfected HC11 cells were determined by measuring invasion through a barrier of reconstituted basement membrane Matrigel over a 72-h period (Fig. 5). The invasive potentials of NIH3T3 mouse fibroblasts and NIH3T3 cells transfected with the wild-type c-fms gene (NIH3T3-FMS) were also tested to provide negative and positive controls. The invasion activity of the HC11 clone expressing wild-type Fms increased 100-fold over the activity of the parental cells (Fig. 5). The HC11 clone expressing Fms mutated at autophosphorylation site Tyr721 also showed an increase in invasive activity similar to the transfectant cells expressing the wild-type Fms, whereas the transfectants expressing Fms mutated at Tyr807 had significantly reduced ability to invade Matrigel. The invasive activity of NIH3T3 cells was similarly enhanced by expression of Fms. We also asked whether exogenous CSF-1 (100 ng/ml) could alter invasiveness of these cell lines but found no measurable effect on the invasion potential of any cell line studied (data not shown).

Effect of CSF-1R on Proteolytic Enzymes Secreted by HC11 Cells. In macrophages and malignant cells of the breast, lung, and ovary, the activity of the uPA is strongly correlated with the ability to invade and metastasize (17, 35). Zymographic analysis for uPA using casein- and plasminogen-impregnated SDS-polyacrylamide gels (Fig. 6A) revealed a prominent lysis zone corresponding to mouse uPA (Mr, 48,000) in conditioned media from cultures of HC11 transfectants with high invasive activity (HC11-FMS and HC11-FMS721) but not in conditioned medium from HC11-FMS807 cells.

The secretion of uPA by HC11 clones was quantitated using a chromogenic microtiter plate assay for uPA (Table 2). This assay is very sensitive, detecting as little as 0.01 mIU of enzyme, and allows discrimination between uPA and tissue-type plasminogen activator (30). Conditioned media from HC11 clones expressing either the wild-type or Tyr721-mutated Fms contain significant amounts of uPA (565 and 360 mIU, respectively), whereas conditioned media from HC11 and HC11-FMS807 cells had no measurable uPA activity.
Table 1: Quantitation of anchorage-independent growth for HC11 clonal cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of colonies</th>
<th>Matrigel</th>
<th>Matrigel (150 μg/ml)</th>
</tr>
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<tbody>
<tr>
<td>HC11</td>
<td>10^6</td>
<td>1 ± 0.5</td>
<td>1 ± 0.6</td>
</tr>
<tr>
<td>HC11-FMS</td>
<td>10^6</td>
<td>38 ± 6.0</td>
<td>122 ± 14.0</td>
</tr>
<tr>
<td>HC11-FMS807</td>
<td>10^6</td>
<td>25 ± 8.3</td>
<td>92 ± 19</td>
</tr>
<tr>
<td>HC11-FMS721</td>
<td>10^6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values represent means ± SE for three independent experiments; six wells/cell line were used in each experiment.

Fig. 4. Soft agar colony assay for anchorage-independent growth. Untransfected or wild-type or mutated FMS-transfected HC11 cells were grown in 0.3% soft agar culture in the absence (A) or presence (B) of basement membrane matrix Matrigel. Photographs represent typical regions of the cultures.

Taken together, our data show that the level of uPA activity measured by the zymographic assay (Fig. 6A) and the quantitative uPA assay (Table 2) correlates well with the invasion capacity of the HC11 clones (Fig. 5).

Degradation of collagen in the extracellular matrix by metalloproteinases is one of the key steps in the invasion of blood vessels and extravasation by tumor cells (36). Acquisition of the ability to degrade collagen is one of the critical steps in the transition from in situ to invasive carcinoma as well as tumor cell intravasation and extravasation (36). Zymographic analysis of the supernatants from the same HC11 clonal cells for metalloproteinases using collagen I-impregnated SDS-PAGE gels (Fig. 6B) revealed the presence of three distinct lysis zones, of apparent Mr 92,000, 83,000, and 48,000, in all cell lines tested, regardless of their invasive ability. All three bands were confirmed to belong to the metalloproteinase family, because their activities were inhibited by incubation of the gel in the presence of EDTA (data not shown). We concluded that these lysis zones corresponded to the previously identified Mr 92,000, 83,000, and 48,000 species of metalloproteinases (35). Interestingly, we observed differences between the HC11 clones expressing Fms mutated at the Tyr807 site and the other HC11 clones in the ratios of the bands (Fig. 6B). In HC11-FMS807 cells, the 92-kilodalton species was more abundant than the 83- and 48-kilodalton forms, suggesting that the CSF-1R may have some regulatory role in the expression of collagenase isoforms.

If uPA and collagenases are essential to the invasion process, an inhibition of invasion should be observed with the addition of specific neutralizing antibodies or protease inhibitors. The invasiveness of the HC11 cells transfected with the wild-type c-fms gene was assessed using the Matrigel invasion assay in the presence or absence of anti-uPA (moAB 394, an effective inhibitor of the enzymatic activity of uPA; Ref. 27) or two metalloproteinase inhibitors (TIMP1 and TIMP2). Invasion by HC11-FMS cells was effectively inhibited by anti-uPA moAB in a dose-dependent manner, whereas nonspecific mouse IgG has no effect on invasion (Fig. 7). TIMP1 (3 μg/ml) reduced the invasion by about 85%, showing inhibition of invasion comparable to moAB 394 (150 μg/ml), whereas the same concentration of TIMP2 (3 μg/ml) attenuated the invasion of this clone by only 60% (Fig. 7). We also tested the possibility that anti-uPA antibody.

Fig. 5. Invasion by different HC11 clones. The columns show the numbers of invading cells from the untransfected HC11 cells or cells transfected with wild-type or mutated Fms. NIH3T3 and NIH3T3-FMS cell lines were also included as controls. Invasion studies were performed by adding 1 × 10^4 cells to the upper part of invasion chambers coated with 1 μg/mm^2 Matrigel. Cells that invaded through the Matrigel were stained and counted after 72 h. The values are means from at least six independent experiments; bars, SE. In each experiment, six filters were used for each cell line.
produce experimental lung lesions after i.v. injection into BALB/c HC11 clones in vivo, we tested HC11 transfectants for their ability to invasion activity of the HC11 cells by anti-uPA, TIMP1, and TIMP2.

We did not find any measurable effect on the growth rate of these cells using a standard proliferation assay (data not shown). We concluded that the observed inhibitory effect on the invasion activity of the HC11 cells by anti-uPA, TIMP1, and TIMP2 was not due to an alteration of the growth characteristics of the HC11 cells.

**Effect of Fms on Experimental Metastasis in Vivo.** To determine the effect of stable expression of Fms on the metastatic activity of HC11 clones in vivo, we tested HC11 transfectants for their ability to produce experimental lung lesions after i.v. injection into BALB/c mice. We injected 2 × 10^5 or 2 × 10^6 cells from the untransfected HC11 clone and from HC11 clones overexpressing either the wild-type or mutated (Tyr807 or Tyr721) Fms into the tail veins of BALB/c mice. Mice were sacrificed 50 days after injection, and the tumor nodules in the lungs were counted. Injection of HC11 transfectant cells expressing the wild-type Fms into BALB/c mice produced a ~10-fold higher incidence of lung tumors than the parental cell line (Fig. 8). HC11 transfectant clones expressing Fms mutated at Tyr807 resulted in 60% fewer lung lesions than the cells expressing the wild-type Fms. However, there were no lung lesions in the mice injected with HC11 transfectant cells expressing Fms mutated at Tyr721.

![Fig. 6. Zymographic analysis of proteinases expressed by the different HC11 clones. Cells (1 × 10^6) were incubated in serum-free medium for 24 h. The conditioned medium from each HC11 clone was mixed with sample buffer and electrophoresed on 10% SDS-PAGE copolymerized either with 1 mg/ml casein and 25 mg/ml bovine plasminogen (A) or with 1 mg/ml rat tail collagen I (B).](image)

![Fig. 7. Inhibition of the invasion of HC11 clonal cells through Matrigel either by anti-uPA antibody or by the specific metalloproteinase inhibitors TIMP1 or TIMP2. Cells (1 × 10^6) were first preincubated for 30 min at 37°C with medium alone (0), with 25 or 150 µg/ml anti-uPA antibody, with one of the metalloproteinase inhibitors (TIMP1 or TIMP2) at 3 µg/ml, or with 150 µg/ml irrelevant murine IgG, then seeded on top of the Matrigel. The cells that invaded through the Matrigel were stained and counted after 72 h.](image)

![Fig. 8. Effect of Fms expression on experimental metastasis in vivo. Untransfected HC11 mouse mammary epithelial cells (control) and HC11 cells overexpressing wild-type Fms (HC11-FMS) or mutant Fms (HC11-FMS807 or HC11-FMS721) were tested for their ability to produce experimental lung lesions after i.v. injection into BALB/c mice. The animals were sacrificed 50 days after injection of either 2 × 10^5 or 2 × 10^6 cells, and the lungs were removed. The lungs were fixed in Bouin's solution, and the numbers of the surface colonies were counted under a dissecting microscope. The results shown are the means of three independent experiments; bars, SE.](image)
DISCUSSION

In the studies summarized in this communication, we provide direct evidence of the regulatory function of CSF-1R (Fms) on cellular invasiveness and anchorage-independent growth in mammary epithelial cells. Transfection of the wild-type c-fms gene into a normal, noninvasive mammary epithelial cell line (HC11) resulted in a dramatic stimulation of the invasive phenotype as well as the metastatic potential of these cells, whereas the cellular growth rate was increased only slightly. The stimulatory effect of Fms expression on invasion of the artificial basement membrane by HC11 cells could be inhibited effectively by specific inhibitors of the uPA-collagenase proteolytic cascade. Furthermore, a single-point mutation introduced into one of the major autophosphorylation sites of Fms (Tyr^807) eradicated the stimulatory effect of Fms expression on the invasive ability of HC11 cells and substantially reduced their metastatic potential compared with cells expressing wild-type Fms but did not impair Fms-induced anchorage-independent growth. In contrast, a single mutation at Tyr^721 of Fms had no effect on invasion, as measured in the *in vitro* assay, but markedly attenuated the induction of anchorage-indepen-dent growth by Fms and abolished the metastatic potential of the transfected clones.

In the past, several different model systems have been used to study the role of CSF-1 and CSF-1R in the growth and differentiation of hematopoietic cells and fibroblasts (21, 23, 37). Later, the discovery of CSF-1R expression in malignant epithelial cells (8, 10) raised the question of the role of CSF-1R in epithelial cells. Because the expression and regulation of CSF-1R has been found to be strictly tissue specific (38), we could expect divergence in its function in cells of different origins. In fact, differences between fibroblasts and macrophages in CSF-1R signaling have been described already (20). Therefore, because CSF-1 and CSF-1R are coexpressed in invasive mammary neoplastic cells (15), we studied normal mammary epithelial cells (HC11), which express a high level of CSF-1, as our model system. HC11 cells were isolated from midpregnant BALB/c mouse mammary gland tissue (22) and retained important features of normal differentiation and hormonal responsiveness, as shown by their ability to differentiate and synthesize the milk protein β-casein after stimulation with lactogenic hormones (39). s.c. injection of HC11 cells into nude mice does not produce measurable tumors (22). By transfecting this nontumorigenic and noninvasive mammary epithelial cell line to express CSF-1R, we were able to study the primary role of CSF-1 and CSF-1R in tumorigenesis and invasive ability of epithelial cells.

The correlation of the CSF-1 and CSF-1R autocrine loop in *vivo* with the tumorigenicity of fibroblast and neoplastic epithelial cells was reported previously in two independent studies (21, 40). Introduction of the c-fms gene into BALB/c fibroblasts resulted in efficient transformation: the transfected cells exhibited altered morphology and anchorage-independent growth in soft agar (21). In another report (40), the murine c-fms gene was transfected into a wide range of murine tumor cell lines, which express the ligand CSF-1, and clonogenicity in soft agar was tested. Although the carcinoma-derived cell lines used in this study were already clonogenic, expression of CSF-1R by these cells increased both the number and size of the colonies. Our study is consistent with these findings and supports the role of CSF-1R in cellular transformation; we demonstrated that expression of CSF-1R in an untransformed mammary cell line, which was not able to produce colonies in soft agar, conferred the capacity for anchorage-independent growth. We also demonstrated that mutation at one autophosphorylation site (Tyr^721) of Fms completely abolished the Fms-induced anchorage-independent growth in HC11 mammary epithelial cells, whereas mutation at the Tyr^807 site of Fms had no measurable effect on this end point. Mutation at Tyr^721 of Fms was shown to abolish the binding of the secondary messenger protein phosphatidylinositol 3'-kinase to CSF-1R, whereas mutation at Tyr^807 had no effect on this binding (18). These results are similar to those reported with the dominant-negative mutants of c-fms (41). Expression of Fms 37 (Glu^582 → Lys) and Fms 42 (Asp^776 → Asn) mutant receptors in Rat-2 fibroblasts conferred a dominant loss of Fms-associated phosphatidylinositol 3'-kinase activity as well as specifically inhibited anchorage-independent growth mediated by the normal Fms receptor.

Our finding that transfection of CSF-1R into normal epithelial cells not only impacts on anchorage-independent growth but also results in an invasive phenotype is supported by other investigators studying the invasiveness of neoplastic epithelial cells of the breast, ovary, and lung (16, 17). Those studies demonstrated that exogenous CSF-1 can enhance invasiveness by as much as 6-fold in CSF-1R-positive carcinoma cell lines (16). However these prior studies did not provide any direct evidence for a primary effect of CSF-1R on invasion, because all of the cell lines were already invasive and already expressed significant amounts of CSF-1R. A probable reason for our ability to show a much more dramatic (100-fold) effect on cellular invasion by CSF-1R is that we used a noninvasive, CSF-1R-negative, epithelial cell line as our model. Our invasion experiments with wild-type and mutated Fms transfectant HC11 clones not only allowed us to provide direct evidence of a fundamental role of the CSF1-CSF-1R autocrine loop in the invasiveness of mammary epithelial cells but also helped us define the specific tyrosine motif of the CSF-1R involved in this process. Our experiments with the mutated Fms constructs suggested that the Tyr^807 site of Fms is required for the stimulation of invasion by mammary epithelial cells. Because invasion is a property of differentiated cells, our finding also suggests that the Tyr^807 site might be directly involved in a signal transduction pathway leading to mammary epithelial cell differentiation. This hypothesis is supported by a study demonstrating that the Tyr^807 site of Fms controls CSF-1-dependent differentiation in monocytes (18).

Moreover, the ability of an antibody against uPA to inhibit invasion of the basement membrane by HC11 cells expressing CSF-1R strongly implicates uPA in the invasive phenotype of these cells. These findings are consistent with the results of previous studies on breast, lung, and ovarian carcinoma cell lines (16, 17, 42), which demonstrated that uPA is a primary mediator of exogenous CSF-1-stimulated invasion. Others have also shown that uPA promotes invasion *in vitro* through activation of plasminogen to plasmin, which generates active collagenase, the activity of which further promotes invasion (43). In our study, tissue metalloproteinase inhibitors, potent inhibitors of collagenases (44), also reduced the invasiveness of the cells expressing CSF-1R by 85 and 60% (TIMP1 and TIMP2, respectively). However, our zymographic analysis using a collagen-impregnated gel did not identify a novel or an enhanced lysis zone in cells expressing the wild-type CSF-1R compared with the parental cells. It should be noted that on a zymogram both the latent proenzymes and the cleaved active forms of the enzymes can be visualized, because of the activating effect of SDS. Therefore, we concluded that uPA probably activated a procollagenase that was already present in the parental HC11 cells, and that the addition of TIMP1 or TIMP2 in the invasion assay inhibited the same uPA-collagenase proteolytic pathway at the collagenase level. Because epithelial cells of the mammary gland produce a full spectrum of proteinases during lactogenic differentiation (45), it is not surprising that the original cell line (HC11), isolated from a mammary gland of a midpregnant mouse, was able to synthesize several species of collagenase. It also should be mentioned that the invasion was not entirely inhibited either by uPA antibody alone or by a combination of anti-uPA antibody with TIMP1 or TIMP2 (result not shown). This finding leads to the hypothesis that another matrix-degrading proteinase, such as cathepsin B, a cysteine protease, may also affect invasion (27). Studies are currently in
progress to identify the additional proteolytic enzyme contributing to the enhanced invasion of this cell line expressing CSF-1R.

To study the effect of Fms expression on experimental metastasis, we used the in vivo system of injecting cells into the tail veins of mice, which introduces the cells directly into the circulatory system. Although mutation at Tyr807 probably reduces the metastatic capacity of HC1 cells expressing the mutant receptor by disrupting induction of the uPA proteolytic pathway, mutation of Tyr721 appears to abolish colony formation on the lung. Our results demonstrate that the enhanced incidence of lung tumors after injecting mice with HC1 mammary epithelial cells expressing Fms requires both autophosphorylation and transduction pathways, future experiments defining the molecular nature of these pathways could help further explain how growth factors and their receptors regulate cellular invasion and anchorage-independent growth and could suggest potential molecular targets for these receptors for the design of agents that block specific signaling pathways without otherwise perturbing the normal cellular metabolism.

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REFERENCES


Independent Regulation of Invasion and Anchorage-independent Growth by Different Autophosphorylation Sites of the Macrophage Colony-stimulating Factor 1 Receptor

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