Independent Regulation of Invasion and Anchoragel-dependent 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 placental trophoblasts 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$^{120}$ → Phe and Tyr$^{121}$ → Phe$^{721}$). Mutations at Tyr$^{120}$ eradicated the stimulatory effect of Fms expression 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 Tyr$^{721}$ 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 placental 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 tumour 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 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$^{120}$ → Phe$^{120}$ or Tyr$^{120}$ → Phe$^{121}$). Previous analysis of such phosphorylation site mutants showed that Tyr$^{120}$ and Tyr$^{121}$ play important roles in coupling the CSF-1R to intracellular signal transduction pathways (18, 19). The Tyr$^{120}$ site in particular was demonstrated to be crucial for CSF-1R-dependent monocyte differentiation (18), whereas the Tyr$^{121}$ site of the CSF-1R is required for CSF-1-dependent mitogenesis (20). By expressing CSF-1R mutants with Tyr$^{120}$ and Tyr$^{121}$ play important roles in coupling the CSF-1R to intracellular signal transduction pathways (18, 19). The Tyr$^{120}$ site in particular was demonstrated to be crucial for CSF-1R-dependent monocyte differentiation (18), whereas the Tyr$^{121}$ site of the CSF-1R is required for CSF-1R-dependent mitogenesis (20). By expressing CSF-1R mutants with Tyr$^{120}$ and Tyr$^{121}$ play important roles in coupling the CSF-1R to intracellular signal transduction pathways (18, 19). The Tyr$^{120}$ site in particular was demonstrated to be crucial for CSF-1R-dependent monocyte differentiation (18), whereas the Tyr$^{121}$ site of the CSF-1R is required for CSF-1R-dependent mitogenesis (20).

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 mouse macrophage cell line 2F5 (also known as BAC1.2F5) was cloned by Morgan et al. (23).
Cell lines were propagated at 37°C, with 5% CO2 in DME/F12 medium (Sigma) supplemented with 10% fetal bovine serum (NH33T3-FMS) or with 10% fetal bovine serum plus 100 ng/ml recombinant human CSF-1 (2F5). HC11 cells were never allowed to exceed ~60% confluency in DME/F12 medium (Sigma) supplemented with 10% fetal bovine serum, 5 μg/ml bovine insulin (Sigma Chemical Co.), and 10 ng/ml murine epidermal growth factor (Sigma). Fetal bovine serum and calf serum were generously provided by Cetus Corp. HC11 cells stably transfected with the pcDNA3 expression vector encoding cDNAs for either the wild-type, Tyr→Phe mutation, or Tyr→Phe721 mutation of Fms were maintained in the presence of gentamicin sulfate (300 μg/ml; Gemini Bio-Products). Before harvesting the cells for RNA extraction or zymographic analysis, the culture medium was decanted and replaced with serum-free DME/F12 medium (Sigma) for 24 h.

Soft agar colony assays were carried out in 12-well plates. Cells (104) were plated in 0.3 ml of 0.3% Seaplaque agarose (FMC Bioproducts) in DME/F12 medium (Sigma) supplemented with 10% fetal bovine serum and 0 or 150 μg/ml Matrigel (Collaborative Research). An underlayer of 1% agarose was used to prevent attachment and spread of cells. The number of colonies was scored after 14 days.

A standard proliferation assay was used to measure the cell growth rate as described by Stehau et al. (24). Briefly, 105 cells were plated on Matrigel-coated (1 μg/ml) 96-well microriter plates in DME/F12 medium (Sigma) supplemented with various amounts of NuSerum (synthetic serum; Collaborative Research) or fetal bovine serum (Life Technologies) as depicted in Fig. 3. Cultures were incubated at 37°C in 5% CO2 for 72 h. Cells were then fixed with 10% trichloroacetic acid (J. T. Baker) for 30 min at 4°C. Plates were washed several times with distilled water and air dried at room temperature. Trichloroacetic acid-fixed cells were stained with 1% (w/v) bromophenol blue (Bio-Rad) in methanol for 20 min at room temperature. Unbound dye was quickly removed by washing the plates several times with 1% acetic acid, and the plates were air dried overnight. Bound dye was solubilized by adding 100 μl of 10 mM unbuffered Tris base to each well and shaking for 5 min. Solubilized dye was quantitated by measuring absorbance at 590 nm using a Microplate reader (Dynatech Laboratories, Inc.).

Cloning cDNAs Encoding the Wild-Type or Mutated Mouse CSF-IR. The wild-type murine c-fms cDNA clone was excised from the pMZen (c-fins) Trichloroacetic acid-fixed cells were stained with 1% (w/v) bromophenol blue epidermal growth factor (Sigma). Fetal bovine serum and calf serum were generously provided by Cetus Corp. HC11 cells stably transfected with the pcDNA3 expression vector encoding cDNAs for either the wild-type, Tyr→Phe mutation, or Tyr→Phe721 mutation of Fms were maintained in the presence of gentamicin sulfate (300 μg/ml; Gemini Bio-Products).
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 DMEM/F12 medium (Sigma) containing 0.1% BSA into the inserts. Cells (5 × 10⁵) suspended in DMEM/F12 medium (Sigma) containing 0.1% BSA were then seeded into the upper chamber in 200 μl of medium, and DMEM/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 moAB 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 DMEM/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 (10⁶, 10⁷, or 10⁸ 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 Ruw 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 lungs were counted using a dissection microscope. Groups of mice injected with 2 × 10⁶ and 2 × 10⁷ 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-IR.** 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 transfected 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.

Western blot analysis of the cell lysates of the HC11 cells transfected with the wild-type or point-mutated c-fms revealed that the cells express detectable levels of CSF-1R (Fig. 2, A, Lanes 2 and 3, and B, Lanes 1 and 2), corresponding in size to that previously reported for the mature protein (4). However, no detectable CSF-1R was observed in the cell lysate of the parental HC11 cells (Fig. 2A, Lane 1).

**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
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 (in either 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 was 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 transfecant cells expressing the wild-type Fms, whereas the transfecants 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 565 and 360 kDa, respectively), whereas conditioned media from cultures of HC11 transfecants 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 HCII clonal cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Total cells</th>
<th>- Matrigel</th>
<th>+ Matrigel (150 μg/ml)</th>
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<tbody>
<tr>
<td>HCII</td>
<td>10^5</td>
<td>1 ± 0.5</td>
<td>1 ± 0.6</td>
</tr>
<tr>
<td>HCII-FMS</td>
<td>10^5</td>
<td>38 ± 6.0</td>
<td>122 ± 14.0</td>
</tr>
<tr>
<td>HCII-FMS807</td>
<td>10^5</td>
<td>25 ± 8.5</td>
<td>92 ± 19</td>
</tr>
<tr>
<td>HCII-FMS721</td>
<td>10^4</td>
<td>0</td>
<td>0</td>
</tr>
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4 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 HCII 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.

Inhibition of invasion should be observed with the addition of specific neutralizing antibodies or protease inhibitors. The invasiveness of the HCII cells transfected with the wild-type csf-1r 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 HCII-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,
mice. We injected $2 \times 10^5$ or $2 \times 10^6$ 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 transfecant 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 transfecant 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 transfecant cells expressing Fms mutated at Tyr721.

**Table 2 Activity of uPa secreted by HC11 clones**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>uPa activity (mIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC11</td>
<td>0</td>
</tr>
<tr>
<td>HC11-FMS</td>
<td>565 ± 85</td>
</tr>
<tr>
<td>HC11-FMS807</td>
<td>360 ± 40</td>
</tr>
<tr>
<td>HC11-FMS721</td>
<td>7800 ± 300</td>
</tr>
<tr>
<td>2F5</td>
<td></td>
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* uPa activity is defined as fibrin-independent and plasminogen-dependent uPa activity. See "Materials and Methods" and Karlan et al. (30). Values represent means ± SE of three independent experiments, with three dishes/cell line in each experiment.

TIMP1, or TIMP2 could alter the growth characteristics of the wild-type or HC11-FMS cells and found no 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 clones.

**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 transfecants for their ability to produce experimental lung lesions after i.v. injection into BALB/c mice. We injected $2 \times 10^5$ or $2 \times 10^6$ 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 transfecant 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 transfecant 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 transfecant cells expressing Fms mutated at Tyr721.

**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 ($1 \times 10^6$) 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. Columns, means from at least four independent assays; bars, SE. In each experiment, six filters were used for each condition, for each cell line.

**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 $2 \times 10^5$ or $2 \times 10^6$ 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.
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 (Tyr807) 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 Tyr721 of Fms had no effect on invasion, as measured in the in vitro assay, but markedly attenuated the induction of anchorage-independent 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 vitro 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 epithelial 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 (Tyr721) of Fms completely abolished the Fms-induced anchorage-independent growth in HC11 mammary epithelial cells, whereas mutation at the Tyr807 site of Fms had no measurable effect on this end point. Mutation at Tyr721 of Fms was shown to abolish the binding of the secondary messenger protein phosphatidylinositol 3'-kinase to CSF-1R, whereas mutation at Tyr807 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 (Glu582 → Lys) and Fms 42 (Asp776 → 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 Tyr807 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 Tyr807 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 Tyr807 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 confirm this hypothesis.

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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 sites (Tyr807 and Tyr721) of the receptor, but the critical step in blocking metastasis may be inhibition of the cellular signals that lead to anchorage-independent growth.

We have demonstrated that expression of CSF-1R induces clonogenic growth and cellular invasiveness in mammary epithelial cells in vitro and enhances experimental metastasis in mice in vivo. The observations that anchorage-independent growth could be abolished by mutation at Tyr807, but not at Tyr721, and, in contrast, the induced in vitro invasiveness could be diminished by mutation at Tyr807, but not at Tyr721, suggest that mutations at these tyrosine sites of Fms generate receptors that are selectively impaired in signal response coupling. These findings imply that anchorage-independent growth and cellular invasiveness, two crucial steps in tumor development, may be independently regulated by separate phosphotyrosine sites of CSF-1R. Because phosphorylation of these specific tyrosines might activate completely separate secondary signal 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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