Enhanced Ovarian Cancer Tumorigenesis and Metastasis by the Macrophage Colony-Stimulating Factor

Eugene P. Toy*, Masoud Azodi†, Nancy L. Folk‡, Christina M. Zito§, Caroline J. Zeiss¶ and Setsuko K. Chambers#

*Department of Obstetrics and Gynecology, University of Rochester, Rochester, NY, USA; †Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, CT 06520-8063, USA; ‡Millipore, 15 Research Park Dr., St. Charles, MO 63134, USA; ¶Department of Pathology, Yale University School of Medicine, New Haven, CT 06520-8063, USA; §Department of Comparative Medicine, Yale University School of Medicine, New Haven, CT 06520-8063, USA; ¶Department of Comparative Medicine, Yale University School of Medicine, New Haven, CT 06520-8063, USA; #Arizona Cancer Center, University of Arizona, Tucson, AZ 85724-5024, USA

Abstract

Coexpression of the macrophage colony-stimulating factor (CSF-1) and its receptor (CSF-1R) in metastatic ovarian cancer specimens is a predictor of poor outcome in epithelial ovarian cancer. This suggests that an autocrine loop is produced by which ovarian tumors can secrete CSF-1 stimulating the CSF-1R resulting in a more aggressive phenotype. Our current work sought to validate this autocrine stimulation model using stable transfection of a 4-kb CSF-1 construct into otherwise nonvirulent Bix3 ovarian cancer cells. A representative clone, Bix3T8.2, produced a 72-fold increase in CSF-1 gene transcription rate (by nuclear run-off assays) and a 57-fold increase in secreted CSF-1 protein (by sandwich ELISA), compared to parent cells. Comparison of Bix3T8.2 invasion, adhesion, and motility in vitro and metastasis in vivo were made to parental and transfectant controls. Up to 12-fold higher invasiveness was seen with Bix3T8.2 and 2- and 6-fold higher adhesion and motility, respectively, over controls in vitro. In nude mice, i.p. injection of Bix3T8.2 produced a wide array of visceral, nodal, and distant metastasis with a degree of enhanced tumor burden not seen in any of the 10 mice inoculated with transfectant control cells. Complete absence of tumor take distinguished 40% of mice implanted with transfectant control cells. Disruption of this autocrine loop using antisense oligomer therapy against CSF-1R and 3′ untranslated region knockdown of CSF-1 protein resulted in reversal of in vitro and in vivo tumor phenotypes. This CSF-1 feedback loop offers a model by which novel biologic therapies can potentially target multiple levels of this pathway.

Neoplasia (2009) 11, 136–144

Introduction

Coexpression of the macrophage colony-stimulating factor (CSF-1) and its receptor (CSF-1R encoded by the proto-oncogene c-fms) has been shown to be a predictor of poor outcome in epithelial ovarian cancer [1]. Clinical specimens from ovarian cancer metastases display strong immunostaining for both CSF-1 and its receptor in contrast to noninvasive borderline tumors, which do not coexpress the CSF-1R/CSF-1, and to benign ovarian tissue, which expresses little to no CSF-1R/CSF-1 [1–3]. At initial clinical presentation, elevated levels of CSF-1 in both serum and ascitic fluid have been correlated...
with poor outcome in these patients with invasive ovarian carcinoma [4,5]. In addition, serum CSF-1 levels have been demonstrated to be a clinically useful correlate to ongoing disease status [6,7].

Given the prognostic value of this secreted form of CSF-1 in the setting of ovarian cancer progression, an in vitro model that characterizes the role of secreted CSF-1 can serve as proof of concept that secreted CSF-1 promotes the activity of ovarian tumor cells. Reiteration of the autocrine loop between the CSF-1 ligand and its receptor provides an experimental model in which the mechanism of ovarian cancer invasion and metastasis can be elucidated. Although the CSF-1 mRNA transcript produces several alternatively spliced products [8], by far the major secreted form of CSF-1 that is found in ascites and serum is encoded by a 4-kb transcript including a 2-kb 3’ untranslated region (UTR). This transcript is regulated posttranscriptionally and is stabilized by mRNA binding proteins (such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) making this 4-kb transcript with its AU-rich 3’UTR the most biologically relevant of the transcripts [9,28].

The ability of ovarian cancer cells to invade a reconstructed basement membrane has been shown to be stimulated by CSF-1 [10]. This stimulation of invasion by the exogenous treatment of ovarian cancer cells seems to be mediated through the actions of the urokinase-type plasminogen activator (uPA) [10]. Urokinase is a serine protease involved in tissue remodeling and, like CSF-1, has been found to be present in elevated levels in many cancers, including those of the breast and ovary, where it is associated with a poor prognosis [11,12]. In our study [11], there was a significant association between ovarian tumors, which coexpress CSF-1/CSF-1R, and those, which coexpress uPA/uPAR. It follows from the clinical correlation of CSF-1 to metastatic progression that uPA is one of the downstream mediators of CSF-1–related tumor behavior.

In the current work, we present the transformation of ovarian cancer cells isolated from ascites by the stable overexpression of the 4-kb CSF-1 and study the effect on phenotypic tumor characteristics both in vitro and in vivo. While characterizing the effect of CSF-1 in these cells, designs for therapeutic targets are also evaluated, which may provide insight into the etiologic role of CSF-1 and the process by which endogenous secretion through overexpression produces an autocrine loop that results in tumorigenicity and metastasis in ovarian carcinoma.

Materials and Methods

Cell Culture

The human ovarian cancer parental cell line Bix3, which is known to secrete relatively low CSF-1 [2,10], was maintained in Dulbecco’s modified Eagle’s medium–F-12 Ham’s medium supplemented with 1% fetal calf serum, 1% penicillin-streptomycin, and 10 μg/ml insulin and transferrin. Transfected Bix3 cell lines were maintained in the same medium but with 200 μg/ml geneticin. Before RNA isolation, the cells were serum-starved for 48 hours. Serum-starved conditioned media were obtained for the parent and Bix3 transfecteds and were concentrated 100-fold using the 10,000 MW cutoff Centrprep 10 concentrators (Amicon, Beverly, MA). Before the invasion assay, the cells were grown in the normal medium but with 1% NuSerum (BD Biosciences, New Bedford, MA) instead of 1% fetal calf serum for 24 hours to limit the presence of protease inhibitors. The invasion assay was carried out in 1% NuSerum for the same reason.

For the experimental model of 3’UTR knockdown of CSF-1 expression, we used the spontaneously immortalized normal ovarian surface epithelial cells (NOSE.1), which secrete low levels of CSF-1 and are nontumorigenic [9], and the Hey ovarian carcinoma cell line, which, in contrast, expresses high levels of CSF-1 and exhibits virulent tumor growth in vitro [9,10,13]. Overexpression of 3’UTR sequences as knockdown of CSF-1 was carried out in these cells to capitalize on these two extremes of CSF-1 expression and tumorigenicity.

Transfection

Cells were cotransfected using Lipofectamine (Gibco BRL, Gaithersburg, MD) with p3ACSF69 (American Type Culture Collection, Rockville, MD) [14], an expression vector containing the 4.0-kb human CSF-1 cDNA sequence, and pWLneo (Stratagene, La Jolla, CA), which contains the neomycin resistance gene and allows for selection by treatment with geneticin. Cells were plated onto 100-mm plates and allowed to grow to 60% confluence. Cells were rinsed twice with PBS and then overlaid with a cocktail of the p3ACSF69, pWLneo, and Lipofectamine in Dulbecco’s modified Eagle’s medium. After a 3-hour or an overnight incubation, the transfection cocktail was removed, and cells were fed with normal media. After a 48-hour recovery period, geneticin (Gibco BRL) was added into the media. Several colonies expressing neomycin resistance were isolated and grown. CSF-1 secretion was measured by CSF-1 sandwich enzyme-linked immunosorbent assay (ELISA) of conditioned media of Bix3 parent and transfected cells, with the highest four transfected clones secreting CSF-1 selected for further characterization. One neomycin-resistant clone that did not secrete any detectable CSF-1 served as a negative control.

CSF-1 Sandwich ELISA

Secrected CSF-1 protein levels were measured in the conditioned medium by CSF-1 sandwich ELISA as described previously [10] and were reported as picograms of CSF-1 per milliliter ± SEM.

Isolation and Analysis of Total Cellular RNA

Total cellular RNA was extracted from Bix3 parent and transfected cells using the guanidium cesium chloride gradient method [15]. The RNA (20 μg per well) were electrophoresed in a 1% agarose-formaldehyde gel and were downward transferred onto Gene Screen Plus (New England Nuclear, Boston, MA). The Northern blots were then hybridized to a 32P-labeled 180-bp exon-1 c-fms probe created by EcoR1 and Hind3 digestion of a Bluescript construct into which exon 1 had been cloned [16]. The blots were washed to a high stringency of 0.1x SSC at room temperature, twice for 30 minutes each with c-fms transcript visualized by autoradiography.

Run-off Transcription Assay

Assays of CSF-1 transcription rate in the nuclei of Bix3 parent and clones were performed as described previously [17], except that the linearized plasmid containing the 779-bp CAT cDNA (pMSGCAT; Amersham Pharmacia, Piscataway, NJ), was included as the negative control.

Invasion Assay

The Membrane Invasion Culture System was used to measure, quantitatively, the degree of invasion of Bix3 parent, Hey parent, NOSE.1, and Bix3 transfected CSF-1–overexpressing clones as a correlate of the phenotypic behavior expected from these respective tumor
cell lines, as described previously [9,10,18,19]. For some experiments, cells were treated with vehicle in the absence or presence of 2 to 10 μM B428, a potent synthetic uPA inhibitor (kindly provided by B.A. Littlefield, Eisai Research Institute, Andover, MA) [20], and invasion was measured through an extracellular matrix barrier. B428 (1 mM) in 10% dimethyl sulfoxide served as the stock solution. Parent or transfected cells, 1 × 10⁶, were seeded onto 10-μm pore filters coated with a human defined matrix containing 50 μg/ml human laminin, 50 μg/ml human collagen IV, and 2 mg/ml gelatin in 10 mM acetic acid. The results are reported as mean percent invasion ± SEM. Four independent experiments were performed.

Assay of Urokinase Activity

Urokinase activity was measured at the cell surface of Bix3 parent cells and transfectants as described [21]. A total of 1.5 × 10⁵ cells per well in complete medium were plated in 96-well plates. The next day, they were 85% confluent. After washing the wells with PBS–0.1% BSA, each well was incubated with 37.5 μg of the chromogenic synthetic uPA substrate S-2444 (Sigma, St. Louis, MO) at 37°C. Wells without cells that had received complete medium only served as the negative control. Absorbance at 405 nm was measured after 6 hours in an ELISA plate reader, with results reported as A₄₀₅ ± SEM. Four independent experiments were performed.

Adhesion and Motility Assays

The Membrane Invasion Culture System was also used to quantitatively measure the degree of adhesion of the Bix3 parent, Hey parent, NOSE.1, and Bix3 transfected clones onto a filter coated with human defined matrix as above. The cells that had implanted on the matrix-coated filter after 2 hours were fixed and stained in 0.5% crystal violet in 25% methanol, and the filters were dried overnight. Crystal violet staining was then extracted from each filter containing adherent cells by incubating in 0.1 M sodium citrate in 50% ethanol twice, and absorbance was read at 585 nm [18,22]. The results were reported as A₅₈₅ ± SEM. Four independent experiments were performed.

Similarly, directed motility assays of Bix3 parent and transfected cells were performed using the Membrane Invasion Culture System, as described previously [18]. Fibronectin (25 μg/ml; Fisher, Pittsburgh, PA) was used as a chemotactant in the lower wells, above which uncoated 10-μm pore filters were placed. The results were reported as mean percent motility ± SEM. Five independent experiments were performed.

c-fms Antisense Therapy

The effect of antisense oligomer therapy for knockdown of the receptor for the CSF-1 ligand endogenously expressed in our clones was also studied. Invasion, adhesion, and motility assays were performed in the presence of a phosphorothioate-modified 18-mer directed toward the c-fms translation initiation site (5′CTGGGCCCATGGCCTCGG′3) and were compared to those of a scrambled 18-mer nonsense control (5′ACCCCGTCTCGGGGCTG′3). Bix3T8.2 cells were transiently transfected with 2.5 to 10 μM of antisense or scrambled oligomers along with Lipofectamine plus reagent (Gibco BRL) with a dose-response curve generated for the individual concentrations after measuring invasion after 72 hours. An optimal concentration of 7.5 μM was initially ascertained from invasion assays using increments of 2.5 μM (data not shown). Three independent experiments were then performed using this ideal oligomer concentration for each of the invasion, adhesion, and motility in vitro tumor assays. Cells were treated for 72 hours with oligomers before performing the in vitro assays.

3′UTR Knockdown of CSF-1

3′UTR sequences are known to regulate most posttranscriptional events and can dramatically alter cellular function by regulating gene expression [23–25]. The 144nt AU-rich element of 3′UTR of the 4-6 shaft CSF-1 described previously [9,26] cloned in a pCMV-Tag1 vector (Stratagene, Santa Clara, CA) was used for stable transfection of the CSF-1–expressing Hey ovarian carcinoma cell line. The vector alone served as a negative control for invasion and in vivo tumor studies in nude mice. Expression of excess 144nt 3′UTR CSF-1 sequences was confirmed in the clones by competitive reverse transcription–polymerase chain reaction, using forward T3 and reverse T7 primers to amplify the cloning site within the pCMV-Tag1 vector, with the empty vector as an internal template for competition. The competitive reverse transcription–polymerase chain reaction was performed as described previously [17].

Intrapерitoneal Tumor Growth in Nude Mice

Tumor burden was measured in vivo 45 days after tumor cell inoculation of Bix3T8.2 CSF-1–overexpressing transfectants or the Bix3T8.3 non–CSF-1–expressing control cells into the peritoneal cavity of 6- to 8-week-old female NIH athymic NCr-nu mice (10 per condition). The period was chosen so that the extent of subclinical disease could be measured, i.e., before abdominal distension or any evidence of clinically apparent disease. It has been previously shown [2] that Bix3 parent cells metastasize widely only after 60 days after tumor cell transplantation. On day 0, 1 × 10⁶ cancer cells in serum-free medium were injected i.p. into each mouse. All the mice appeared healthy at the time of euthanasia on day 45. The extent of macroscopic and microscopic metastases as well as microscopic evidence for invasiveness was assessed and compared between the two groups. Specimens from ovary, uterus, mesovarium, mesometrium, omentum, mesentery, lymph nodes, bowel, spleen, pancreas, liver, portal region, kidney, diaphragm, mediastinum, and lung were collected, fixed in 10% phosphate-buffered formalin, and processed for standard histologic analysis.

Similar i.p. inoculation of 3′UTR knockdown Hey cells and controls was performed in the NIH athymic NCr-nu mice.

Statistical Analysis

Paired or unpaired t tests, or Kruskal-Wallis one-way analysis of variance on ranks was applied, as appropriate, using Sigma Stat (Jandel Scientific Corp., San Rafael, CA). A P value of <.05 was considered statistically significant.

Results

Characterization of CSF-1 Clones

Secreted CSF-1 protein levels. Bix3 parent cells secrete relatively small amounts of CSF-1 into conditioned media compared with other more invasive ovarian cancer cell lines [10]. To initially screen Bix3 transfectants that were selected by neomycin resistance, we evaluated levels of secreted CSF-1 from conditioned media. From four transfected clones that produced similar magnitudes of elevated CSF-1 secretion, we selected one representative CSF-1–producing clone, Bix3T8.2, which produced a 72-fold increase (360 ± 3.1 pg/ml) of secreted CSF-1 when compared to Bix3 parent cells (5.0 ± 0.2 pg/ml).

3′UTR Knockdown of CSF-1

3′UTR sequences are known to regulate most posttranscriptional events and can dramatically alter cellular function by regulating gene expression [23–25]. The 144nt AU-rich element of 3′UTR of the 4-kb CSF-1 described previously [9,26] cloned in a pCMV-Tag1 vector (Stratagene, Santa Clara, CA) was used for stable transfection of the CSF-1–expressing Hey ovarian carcinoma cell line. The vector alone served as a negative control for invasion and in vivo tumor studies in nude mice. Expression of excess 144nt 3′UTR CSF-1 sequences was confirmed in the clones by competitive reverse transcription–polymerase chain reaction, using forward T3 and reverse T7 primers to amplify the cloning site within the pCMV-Tag1 vector, with the empty vector as an internal template for competition. The competitive reverse transcription–polymerase chain reaction was performed as described previously [17].
Invasion. The degree of invasion through an extracellular matrix barrier by the CSF-1–overexpressing transfectant Bix3T8.2 was compared with that of controls where CSF-1 expression and ability for invasion had been quantitatively measured. It has previously been shown that the Bix3 parent cells are weakly invasive through both a Matrigel and a human defined matrix barrier [10]. We confirm our observation that Bix3 cells are minimally invasive (1.12 ± 0.14% invasion) and demonstrate that the stably transfected Bix3T3.1 cells, which do not express CSF-1, are similarly (P > .05) minimally invasive through the human defined matrix (0.79 ± 0.16% invasion). Thus, stably transfected Bix3 cells, which express only the neomycin resistance gene, do not exhibit enhanced invasiveness. In contrast, we find that with the CSF-1–overexpressing transfectant Bix3T8.2 line, the degree of invasion was significantly increased (P < .001) by almost 9-fold (9.73 ± 1.22% invasion), when compared to parent cells, or by 12.3-fold (P < .001), when compared to the neomycin-resistant transfectant control (Figure 2).

To provide evidence that this enhanced invasiveness occurring in the CSF-1–overexpressing Bix3T8.2 cell line was a result of the urokinase system previously described [10], we used the potent synthetic uPA inhibitor B428. B428 [20] has been shown to inhibit cell surface uPA and surface uPA–mediated cellular degradative functions. Compared to vehicle or untreated controls, we show a clear dose–response inhibition of invasiveness in these cells of up to 2.8-fold by increasing doses of B428 from 2 to 10 μM (Figure 3; P = .002). There is no significant effect of the vehicle on invasiveness, compared to untreated cells. Thus, the significant increase in cell surface uPA activity that we demonstrated by CSF-1 seems to contribute in part to the enhanced invasiveness exhibited by Bix3T8.2 cells.

**Adhesion and motility.** The correlation of secreted CSF-1 circulating in serum and ascites with advanced metastatic disease suggests induction of a metastatic cascade after the initial tumor invasion. The ability of the Bix3T8.2-transfected cells to adhere to a membrane coated with human defined matrix was studied in comparison with the Bix3T3.1 non–CSF-1–expressing transfectant line. As seen in Figure 4, the BixT8.2 cells exhibited almost a two-fold increase (P = .007) in the percentage of cells adhering to the membrane

Assessment of Bix3T8.2 Tumor Phenotype

**CSF-1 gene transcription.** To ascertain whether the increased protein production of secreted CSF-1 was a direct result and in proportion to enhanced CSF-1 gene expression in Bix3T8.2 cells, as we would expect in this experimental model of stable transfection, we quantitated the resultant rate of CSF-1 gene transcription from nuclei of Bix3 CSF-1 overexpressing cells. A 26.9-fold increase in CSF-1 gene transcription was demonstrated in the nuclei of Bix3T8.2 CSF-1–overexpressing cells, compared to that in the nuclei of Bix3 parent cells (Figure 1). Relative to the Alu control, which serves as a measure of global transcription and is increased in the Bix3 parent compared to the Bix3T8.2 cells, CSF-1 gene transcription is increased 57.2-fold in the Bix3T8.2 nuclei. The pMSGCAT vector sequences serve as the negative control for gene transcription. Thus, CSF-1 overexpression seen in the Bix8T8.2 clone–transfected clones seems to be largely on the basis of enhanced CSF-1 gene transcription because the increase in CSF-1 mRNA transcription and secreted protein is of a similar magnitude.

**Invasion.** The degree of invasion through an extracellular matrix barrier by the CSF-1–overexpressing transfectant Bix3T8.2 was compared with that of controls where CSF-1 expression and ability for invasion had been quantitatively measured. It has previously been shown that the Bix3 parent cells are weakly invasive through both a Matrigel and a human defined matrix barrier [10]. We confirm our observation that Bix3 cells are minimally invasive (1.12 ± 0.14% invasion) and demonstrate that the stably transfected Bix3T3.1 cells, which do not express CSF-1, are similarly (P > .05) minimally invasive through the human defined matrix (0.79 ± 0.16% invasion). Thus, stably transfected Bix3 cells, which express only the neomycin
(66.9 ± 6.7%) compared with the parental Bix3 cells (35.8 ± 2.8%) and the negative Bix3T3.1 control (47.3 ± 2.3%).

Further study of the metastatic potential of these Bix3T8.2 cells was performed using the same chamber assay now with fibronectin as a chemoattractant to determine directed motility of the tumor cells through the membrane. As seen in Figure 5, the BixT8.2 cells exhibited an approximately six-fold increase \((P < .001)\) in the percentage of cells (18.0 ± 1.9%) migrating through the membrane in the absence of matrix components to the lower chamber compared with the parental cells (3.1 ± 0.4%) and the Bix3T3.1 control (3.7 ± 0.3%). Again, a highly significant statistical difference was seen between the groups \((P < .001)\). No significant differences were seen in either adhesion or motility assays between the parental negative control and the transfectant Bix3T3.1 control.

\[\text{Figure 3. Degree of invasiveness through a human defined matrix of CSF-1–overexpressing Bix3T8.2 cells in the presence or absence of B428, a potent uPA inhibitor (2-10 \(\mu\)M). The cells that invaded through the human defined matrix–coated filter after 48 hours were stained, counted, and expressed as mean percent invasion ± SEM of the total number of cells loaded into the upper chamber with significant inhibition of invasiveness in a dose-response fashion seen with B428 \((P = .002)\).}\]

\[\text{Figure 4. Degree of adhesion of Bix3 parent and transfected cells 2 hours after seeding on a human defined matrix. Bix3T3.1 cells served as the non–CSF-1–expressing transfectant control. Absorbance was measured at 585 nm after the extraction of crystal violet from stained adhered cells and expressed as mean } A_{585} \pm \text{SEM (bars). A two-fold increase in adhesiveness was seen with Bix3T8.2 over controls } (P = .007).\]

\[\text{Figure 5. Degree of fibronectin-directed motility of Bix3 parent and transfected cells. Bix3T3.1 cells served as the non–CSF-1–expressing transfectant control. The cells that moved from the upper to the lower fibronectin-containing chamber after 6 hours were stained, counted, and expressed as mean percentage of the total number of cells loaded into the upper chamber ± SEM (bars). Effect of antisense treatment of cells in the presence of 7.5 \(\mu\)M of an 18-mer directed antisense toward the } c-fms \text{ translation initiation site or its scrambled oligomer control for 72 hours is also shown (shaded half). This difference in motility was highly, statistically significant } (P < .001).\]

\[\text{In vivo metastasis. We thus have shown that endogenous CSF-1 overexpression in ovarian cancer cells leads to increased invasiveness, adhesion, and motility, all crucial factors in the metastatic cascade. We next studied the role for CSF-1 overexpression directly on the metastatic phenotype of ovarian cancer cells, in nude mice.}\]

\[\text{Four of the 10 control mice bearing non–CSF-1–expressing Bix3T3.1 cells had no pathologic evidence for cancer. The rest had one to three foci of 0.5- to 1-mm peritoneal implants. In contrast, 9 of the 10 mice bearing CSF-1–overexpressing Bix3T8.2 cells had several, up to 20, 2- to 5-mm implants throughout the peritoneal cavity. The remaining mouse had evidence for extensive lymph node metastases. On microscopic examination of the 6 of the 10 mice that developed tumor from the non–CSF-1–expressing Bix3T3.1 ovarian cancer cells, 3 had evidence for multifocal peritoneal disease and the other 3 had evidence for unifocal peritoneal disease. Their nodules were well circumscribed, were enclosed in a fibrous capsule, and were accompanied by a nonsuppurative inflammatory infiltrate. None of these 6 mice had any evidence for lymph vascular space invasion, lymph node metastasis, invasion of the ovary or any other organ, transdiaphragmatic invasion, or distant metastasis (Table 1).}\]

\[\text{Table 1. Metastasis in Nude Mice Related to CSF-1.}\]

<table>
<thead>
<tr>
<th>Tumor Site</th>
<th>Transfectant Cell Lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bix3T3.1 ((N = 10))</td>
</tr>
<tr>
<td>Peritoneal seeding</td>
<td>6 9</td>
</tr>
<tr>
<td>Visceral ovarian involvement</td>
<td>0 4</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>0 8</td>
</tr>
<tr>
<td>Distant lung (including transdiaphragmatic invasion)</td>
<td>0 4 (5)</td>
</tr>
<tr>
<td>Total number of mice exhibiting tumor</td>
<td>6/10 10/10</td>
</tr>
</tbody>
</table>

\[\text{Widely metastatic disease was seen after intraperitoneal injection of CSF-1 overexpressing Bix3T8.2 cells compared to control Bix3T8.2.}\]
In marked comparison, all 10 mice bearing the CSF-1–overexpressing Bix3T8.2 ovarian cancer cells had evidence for either extensive peritoneal spread of tumor and/or extensive lymph node metastases (Table 1). Further, organ invasion and distant metastasis were common findings. The portal region was involved by tumor in five mice, visceral invasion of the ovary in four mice (visceral invasion of the liver and pancreas was less frequently observed), transdiaphragmatic invasion in three mice, lymph node invasion in eight mice (most commonly affected lymph nodes were mesenteric, portal, inguinal, mediastinal, and rarely submandibular; lymph vascular space invasion was present in animals with extensive tumor infiltration), and lung metastases in four mice. These metastases were unencapsulated, invasive, and did not evoke an inflammatory infiltrate. Representative examples of differences in the microscopic appearance of tumor developing after i.p. injection of Bix3T3.1 versus T8.2 cells are depicted in Figure 6.

Therefore, we have demonstrated a marked increase in i.p. tumor burden, lymph node and organ invasiveness, and distant metastasis, resulting from CSF-1–overexpressing transfectants when compared to their non–CSF-1–expressing controls. Further, our data suggest an effect of CSF-1 on tumorigenicity, in that 40% of mice bearing non–CSF-1–expressing cells did not develop any tumors, in comparison to the virulence of tumors developing in 100% of mice bearing Bix3T8.2 CSF-1–overexpressing cells.

 Establishment of the Autocrine Loop Pathway as a Mechanism for CSF-1 Transformation of Bix3 Ovarian Carcinoma Cells

Knockdown of the CSF-1 receptor. Confirmation of CSF-1 autocrine stimulation in the Bix3T8.2 tumor cells was further provided by the interruption of the autocrine loop using antisense therapy directed against the CSF-1R. Northern blot analysis of antisense-treated Bix3T3.1 cells is shown in Figure 7. Successful down-regulation of c-fms proto-oncogene expression is seen compared with the nonsense oligomer treatment and Lipofectamine alone. Similar results were demonstrated in Bix3T8.2 cells (data not shown).

With this antisense inhibition of the c-fms proto-oncogene encoding the CSF-1R, we performed parallel experiments to study invasion, adhesion, and motility in the CSF-1–overexpressing transfectant.

Figure 6. Comparison of extent and histologic characteristics of tumor metastases between nude mice bearing the control Bix3T3.1 (A, C) or CSF-1–overexpressing Bix3T8.2 (B, D) ovarian cancer cells. (A) Bix3T3.1. Omentum. Nodular neoplastic masses (asterisks) are encapsulated by fibrous tissue and are accompanied by a (nonsuppurative) inflammatory infiltrate (thick arrow). Single neoplastic cells are seen infiltrating the surrounding fibrous tissue (thin arrow). Bar, 200 μm. (B) Bix3T8.2. Mesometrium (mouse equivalent of peritoneum supporting the ovary) metastasis. Neoplastic nodules are unencapsulated and do not evoke an inflammatory reaction, indicating more rapid metastasis than in (A). Bar, 200 μm. (C) Bix3T3.1. Diaphragm. Metastatic cells are limited to a heavily encapsulated nodule located on the peritoneal surface of the diaphragm. Bar, 200 μm. (D) Bix3T8.2. Diaphragm. Large neoplastic masses extend from the peritoneal surface (asterisk) through the diaphragm to the pleural surface (arrow). Bar, 500 μm.

Figure 7. Northern blot analysis of antisense treatment of c-fms showing knockdown of gene expression. The blot was hybridized to a 32P-labeled 180-bp exon-1 c-fms probe created by EcoRI and Hind3 digestion of a pBluescript construct into which exon 1 had been cloned [17]. Autoradiography reveals decreased c-fms transcript by antisense treatment.
Bix3T8.2 cell line. Shown in the shaded area of Figure 2, the antisense treatment of the CSF-1–overexpressing Bix3T8.2 cells produced approximately a 3-fold reduction of the percent invasion (3.37 ± 0.42%) compared with the untreated Bix3T8.2 cells and a 3– to 3.5-fold reduction compared with the Lipofectamine- (10.5 ± 1.4%) and nonsense- (11.3 ± 1.7%) treated Bix3T8.2 cells, respectively (P < .001). Of note, there was no significant difference between the invasiveness of Bix3T8.2 cells with antisense c-fms treatment and Bix3T3.1 control, or Bix3 parent cells. Similar results were seen in the constitutively CSF-1–overexpressing parental Hey ovarian carcinoma cell line with a two-fold reduction in invasion (5.83 ± 1.01%) observed with antisense treatment compared with the nonsense control group (10.44 ± 0.92%). In a parallel adhesion assay using Bix3T8.2 cells, almost a three-fold decreased adhesion was seen (P = .011) using antisense therapy compared to the application of nonsense oligomers (data not shown). The decrease in motility translated into an approximate 2-fold reduction, again, in the antisense treatment group (10.3 ± 1.2) compared with the untreated parental cells and a 1.5– to 1.7-fold decrease compared to the nonsense- and Lipofectamine-treated groups (P < .001; Figure 5, shaded). There were no significant differences seen between the negative control groups within the various assays. Thus, interrupting the autocrine loop by antisense targeting of the CSF-1R resulted in a substantial inhibition of the invasive, motile, adhesive ovarian cancer phenotype.

3′UTR knockdown of CSF-1. To further study the interruption of the autocrine loop but at the feedback level of secreted CSF-1 protein, we introduced an expression plasmid containing cDNA corresponding to the 144nt AU-rich fragment of the 3′UTR of the 4-kb CSF-1 mRNA by stable transfection into the notably tumorigenic and virulent CSF-1–overexpressing Hey ovarian carcinoma cell line [10,14], as well as the spontaneously immortalized NOSE.1 cells [9]. Most posttranscriptional events are regulated by 3′UTR sequences. The 3′UTRs have the capacity to dramatically alter cellular function by regulating gene expression [23–25]. These actions of 3′UTR RNA are thought to be modulated by RNA protein binding. We focused on this 144nt AU-rich element [9,26] because we had previously defined this region as critical for regulatory protein binding of the 4-kb CSF-1 transcript. In fact, we have previously shown that excess 144nt AU-rich sequences can downregulate endogenous CSF-1 mRNA levels [9]. Further, we have demonstrated that GAPDH protein binding with this 3′UTR element promotes CSF-1 mRNA stability and that silencing GAPDH decreases secreted CSF-1 levels in NOSE.1 and Hey cells [26]. Delivery of excess 3′UTR sequences seems to act by sequestering stabilizing proteins such as GAPDH, resulting in the down-regulation of gene expression.

We first studied the effect of excess 144nt 3′UTR CSF-1 sequences on secreted CSF-1 levels. More than a two-fold reduction (P < .001) in the secretion of CSF-1 was seen from parental Hey cells (791 ± 26 pg/ml) compared to the 3′UTR expressing transfectant (387 ± 19 pg/ml), and this mirrored the reduction seen in the spontaneously immortalized NOSE.1 control cell line, which was also on the order of a 1.5– to 2-fold reduction compared to parental- and vector-alone controls (data not shown).

With CSF-1 levels reduced by half from the parental cells, we assessed tumor phenotype using both in vitro invasion/motility assays and the measurement of in vivo metastasis. First, a baseline comparison of our transfectant-negative control Bix3T3.1, immortal NOSE.1-negative control, the CSF-1–overexpressing Bix3T8.2 transfectant, and positive control Hey parental cells was made for both invasion and motility. A 10-fold increase in the degree of invasion was seen with Hey cells compared to NOSE.1 cells with an almost three-fold increase in motility seen (Figure 8). Hey cells provided the extreme range of invasiveness and motility in this in vitro model (P < .001; Figure 8). With the overexpression of the 144nt 3′UTR CSF-1 sequence in NOSE.1 and Hey cell lines, a significant reduction in invasiveness through human defined matrix compared to parent or vector controls was seen in both the NOSE.1 cells (2.45-fold) and the Hey cell line (3.25-fold), respectively, with no significant difference detected between vector and parent controls. In Hey cells, the percent invasion of parent cells, 55.5 ± 1.3%, was reduced to 17.1 ± 0.8% (P < .001) in the presence of excess 144nt CSF-1 sequences. The magnitude of reduction of invasiveness of these cells was in line with the level of reduction of secreted CSF-1 by excess 144nt 3′UTR AU-rich CSF-1 sequences. These results provided a basis for our subsequent experiments in nude mice. Having afforded the most extreme range of tumor invasion and motility, we used the Hey cells (NOSE.1 cells not being tumorigenic [9]) for our nude mice experiments with 10 mice inoculated in each group. Only 3 of the 10 mice in the 144nt AU-rich 3′UTR CSF-1–overexpressing group manifested any evidence of tumor take by day 45 after inoculation, and these lesions were inoculation site tumors only. This was compared to the 90% of the animals in the untreated Hey group that developed gross evidence of tumors within this time frame. Seven of the 10 animals in the untreated Hey group developed inoculation site tumors, 6 animals developed intra-abdominal tumors, 3 animals showed lymph node invasion, and 2 showed distant spread of tumor to other organ sites (Table 2). There were little differences seen in the pattern of spread between control Hey cells expressing vector sequences and the parent Hey cells (Table 2). This reduction in both tumorigenesis

![Figure 8](image-url). Baseline comparison of motility and invasion of cells used for in vivo studies. Transfected Bix3 and parental NOSE.1 and Hey ovarian carcinoma cells were studied in preparation for i.p. injection into nude mice. An increase of approximately 10-fold greater invasiveness was seen with Hey cells over the nonvirulent NOSE.1 cell line with three-fold greater motility seen in vitro (P < .001).
CSF-1 and Metastasis in Ovarian Cancer  Toy et al. 143

Table 2. 3'UTR Knockdown of CSF-1—Overexpressing Hey Cells in Nude Mice.

<table>
<thead>
<tr>
<th>Tumor Site</th>
<th>Hey Ovarian Carcinoma Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parent  Vector Alone  3'UTR 144nt</td>
</tr>
<tr>
<td></td>
<td>(N = 10) (N = 10) (N = 10)</td>
</tr>
<tr>
<td>Peritoneal seeding</td>
<td>7  6  3</td>
</tr>
<tr>
<td>Visceral ovarian involvement</td>
<td>5  5  0</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>3  0  0</td>
</tr>
<tr>
<td>Distant lung (including transdiaphragmatic invasion)</td>
<td>2  2  0</td>
</tr>
<tr>
<td>Total number of mice exhibiting tumor</td>
<td>9/10 5/10 3/10</td>
</tr>
</tbody>
</table>

Treatment of Hey ovarian carcinoma cells using 3'UTR 144nt AU-rich fragment results in the attenuation of tumor metastasis compared to parent and vector constructs.

and metastatic potential provided additional evidence of the etiologic role of CSF-1 in the progression of ovarian cancer.

Discussion

The prognostic value of CSF-1 in predicting outcome in epithelial ovarian cancer has been recognized [1,4,5]. This cytokine, normally associated with monocyte/macrophage proliferation and placental trophoblast implantation, is also present in cancers of varying origin and is prognostic of invasive tumors and poor outcome. Most, if not all, ovarian cancer cell lines express CSF-1, with the degree of expression correlated with invasive capacity [2,10]. In this report, we describe our finding that Bix3 ascitic human ovarian cancer cells transfected with the 4.0-kb CSF-1 cDNA sequences were transformed into a significantly more invasive, motile, and metastatic counterpart, which largely explains, for the first time, the poor prognosis associated with tumors that overexpress c-fms and CSF-1 in their metastases [1].

Modulation of CSF-1 levels in the Bix3 parent cells was achieved at the gene and protein levels and correlated with tumor phenotype. The low level of CSF-1 expression in these parent cells provides a baseline for both CSF-1 secretion and a tumor behavior on which experimentally enhanced CSF-1 gene transcription can elevate levels of endogenously secreted protein to mimic the pathologic levels found in clinical studies [4]. In contrast, the high-level CSF-1–expressing Hey ovarian cancer cell line provides a benchmark for CSF-1 secretion and extreme invasiveness and virulence of tumorigenicity [9,10] (Figure 8 and Table 2).

This increase in CSF-1 expression (a 72-fold higher gene transcription corresponding to a 57-fold increase in secreted CSF-1 protein production) in the Bix3T8.2 cells resulted in a highly significant increase in the invasiveness, adhesion, and motility observed in vitro (Figures 2, 4, and 5). It is acknowledged that no single in vitro test to assess invasion or motility is sufficient for clinical translation and that the in vitro models used here lacked the advantages of videomicroscopy and three-dimensional modeling used in other models [27,28]. However, clinical evidence of increased tumorigenicity and of its virulence is provided from the in vivo inoculation of nude mice through i.p. injection. This level of tumor take and metastasis is comparable to the baseline tumorigenicity of the aggressive Hey cell line (Table 2). Because the peritoneal circulation is the main route of spread through this route of inoculation, the ability of the overexpressing CSF-1 clone Bix3T8.2 to produce widespread metastasis that is diverse and infiltrative lends credence to the effect of CSF-1 overexpression on the phenotype observed in vivo.

It follows that therapeutic intervention at the mRNA and protein levels of expression should inhibit CSF-1–induced transformation of cells and diminish both invasiveness and metastatic potential. Successful down-regulation of CSF-1 mRNA transcript and secreted protein through interference with the stabilization of the mRNA transcript by silencing of the CSF-1 RNA binding protein, GAPDH [26], is evidence that manipulation of posttranscriptional gene expression is possible and may prove to affect tumor phenotype. Here, we have used the 144nt AU-rich element of 3'UTR CSF-1, which we have shown to be a target for binding by regulatory proteins, for competition with endogenous 3'UTR CSF-1 mRNA to sequester binding partners that would otherwise cause mRNA stabilization, such as GAPDH. AU-rich elements are the best characterized among the variety of signals that dictate mRNA decay.

The effect of overexpression of this 144nt sequence from the CSF-1 transcript is proportionately reflected by the two-fold decrease in secreted CSF-1 levels, resulting in a 2.5- to 3-fold reduction in invasiveness. This then resulted in a disproportionate and dramatic reduction in in vivo tumorigenicity and invasive metastasis (Table 2), which may reflect the critical involvement of the tumor microenvironment on the resulting tumor phenotype. Notably, in our stable transfection model, those metastatic foci arising from ovarian cancer cells not expressing detectable CSF-1 (Bix3T3.1 cells) elicited an intense inflammatory infiltrate and were well encapsulated in a fibrous capsule. This is in striking contrast to the invasive and extensive nature of the metastatic foci arising from the CSF-1–overexpressing Bix3T8.2 ovarian cancer cells, which were not associated with an inflammatory reaction (Figure 6). Interestingly, this in vivo data reflecting the potential protective role of stromal infiltration in the ovarian cancer is in line with our observations in human ovarian cancer specimens of stromal CSF-1 expression portending an improved clinical outcome [1]. This contrasts with the well-accepted tumor-promoting role of tumor-associated macrophages expressing CSF-1 in breast cancer [29]. It is also important to recognize that the effect of excess 144nt AU-rich 3'UTR CSF-1 sequences is not specific to the regulation of endogenous CSF-1 expression and, in sequestering other regulatory RNA binding proteins, could affect the regulation of other targets affecting tumor phenotype. Current and developing in vivo siRNA [30] and miRNA technologies may afford a more efficient and selective targeting of the CSF-1 transcript [31].

Although decreases in secreted CSF-1 protein availability translate into an inhibition in the invasive and metastatic phenotype, disruption of the receptor/ligand autocrine loop provides additional steps to prevent the transformation of otherwise indolent Bix3 parental cells. When stably transfected with the CSF-1 transcript, the degree of mRNA expression and the increased protein production in parental Bix3 correlate with a significantly higher number of invasive cells in vitro and metastatic lesions, in vivo (Figure 2 and Table 1). Anti-sense targeting of the CSF-1R encoded by c-fms results in disruption of this autocrine loop established by the endogenous secretion of CSF-1 from the Bix3T8.2 transfectants, resulting in a significant inhibition of the highly aggressive ovarian cancer phenotype.

Therapeutic application of technologies directed against this autocrine pathway has begun to show promise. The obstacles associated with the delivery of such antisense oligomers have been recently addressed [32]. Elucidation of downstream signaling pathways of CSF-1R [33–36] and other tyrosine kinase receptors has also been investigated; however, despite a number of agents that have been developed, which target c-fms (e.g., vatalanib, ABT869, CYT645, KG20227), to date, none have been tested in ovarian cancer patients [37]. One Food and Drug Administration–approved drug imatinib, which targets tyrosine kinase receptor c-kit as well as c-fms, has failed to show activity in ovarian cancer [38]. Conversely, selective targeting of the uPA pathway, one of the downstream mediators of CSF-1/Csf-1R action, has produced an improvement in progression-free survival of ovarian cancer patients with...
small tumor burden treated in a recent phase 2 trial of Å6 (an uPA peptide that interferes with binding of uPA with uPAR) [37]. The model of autocrine stimulation represents a more biologically relevant system by which ovarian cancers can stimulate invasion and metastasis from sentinel lesions. Current use of microarrays to assess tumor profiles provides only a single level of therapy that siRNA, miRNA, or antisense treatment addresses. The synergistic strategy of biologic therapy, which can also intervene to prevent the secretion of the CSF-1 ligand in addition to targeting of the receptor, seems to offer the most promising approach to interrupting the metastatic cascade, which results from the establishment of the CSF-1 autocrine loop. Currently available biologic therapies for other tyrosine kinase receptor proteins recapitulate this syglogism.

References


