Macrophage Colony-stimulating Factor Mediates Invasion of Ovarian Cancer Cells through Urokinase

Setsuko K. Chambers, Yixun Wang, Robert E. Gertz, and Barry M. Kacinski

Departments of Obstetrics and Gynecology [S. K. C., Y. W., R. E. G., B. M. K.] and Therapeutic Radiology [B. M. K.], Yale University School of Medicine, New Haven, Connecticut 06520-8063

ABSTRACT

The macrophage colony-stimulating factor (CSF-1) is best known as a hematopoietic cytokine important to macrophage activation. Recently, the importance of CSF-1 and its receptor (encoded by the c-fms proto-oncogene) in epithelial ovarian cancer has also been recognized, with overexpression of CSF-1 denoting poor prognosis in ovarian cancer patients. During macrophage activation, CSF-1 promotes urokinase-type plasminogen activator (uPA) activity; in macrophages and in malignant cells of lung, breast, colon, and prostatic origin, uPA activity is strongly correlated with the ability to invade and, in the malignant cells, to metastasize. While there is clear evidence of CSF-1 and uPA expression in primary and metastatic ovarian cancer, the significance of their expression to invasion of these cells has not been explored. We find that all of our ovarian cancer cell lines which we have studied co-express CSF-1 and uPA transcripts and protein. Urokinase expression in these ovarian cancer cell lines correlates with the degree of tumorigenicity in nude mice, with the most virulent tumor resulting from Hey cells, a strong expressor of uPA. We studied the invasion of these primary and established ovarian cancer cells through a Matrigel (reconstituted basement membrane matrix) barrier. The ability of ovarian cancer cells to invade is strongly correlated with endogenous CSF-1 expression (Pearson's correlation, r = 0.91; P = 0.01). A total of 0.90 ± 0.16% of Bix3 cells (very weak expressor of CSF-1) invaded through the barrier, in contrast to 6.95 ± 0.75% of Hey cells (strong CSF-1 expressor) and 10.44 ± 2.33% of Bx1 cells (the strongest CSF-1 expressor). We studied the ability of two of the cell lines to invade human laminin and type IV collagen (Bix3, a weak invader of Matrigel, and Hey, a strong invader), to determine (a) whether our results on a Matrigel matrix may represent a relevant model for invasion in humans and (b) whether there is a potential confounding effect from the cytokines and proteases in Matrigel. On this human simple matrix, we confirm that Bix3 is a weakly invasive cell line (0.33 ± 0.04% invasion) which contrasted to the strongly invasive Hey cell line (8.51 ± 0.47%). Treatment of Bix3 cells with exogenous CSF-1 stimulates percentage of invasion by 2-fold and results in a similar increase in the level of uPA transcripts and cellular associated uPA antigen. Furthermore, cell surface-bound uPA increased from 74% in the absence of CSF-1 to 100% (fully saturated) in the presence of CSF-1. We show that CSF-1-stimulated invasion of Bix3 cells is completely blocked by anti-uPA antibodies but not by preimmune IgG. Collectively, these data suggest that stimulation of invasion by exogenous CSF-1 is mediated entirely through uPA activity. But uPA expression does not directly correlate with invasion. In fact, inhibition of uPA activity in Hey cells (the strongest uPA expressor) by a saturable amount of anti-uPA antibodies results in only a 50% inhibition of invasion. This suggests that, while uPA activity is associated with extent of intraperitoneal tumorigenicity in nude mice and mediates stimulation of invasion by exogenous CSF-1, it is an important, but not exclusive, mediator of ovarian cancer cell invasion.

INTRODUCTION

The macrophage colony-stimulating factor CSF-1 is best known as a hematopoietic cytokine important to the control of proliferation and invasive differentiation of the macrophage and its monocytic progenitors (1). The actions of CSF-1 are mediated via binding to a tyrosine kinase receptor encoded by the c-fms proto-oncogene (2). During macrophage activation, CSF-1 augments production of active cytokines (such as γ-IFN), as well as the transcript level and enzymatic activity of uPA (3, 4). The invasive properties of the macrophage are believed to be due to uPA activity (5), which confers on macrophages the capacity for localized proteolysis of the surrounding extracellular matrix. Monocytic differentiation of U937 leukemia cells into macrophage-like cells by phorbol esters results in an increase in receptor-bound uPA (6). Results of studies of CSF-1 and its receptor in settings outside of monocytic differentiation (7–17) have paralleled those observed during monocytic differentiation; for instance, in lung carcinoma cell lines, CSF-1 treatment has been shown to increase receptor-bound uPA (8).

Recently, the importance of CSF-1 and c-fms in the biology of human epithelial cancers of ovarian, breast, endometrial, pulmonary, and pancreatic origin has been recognized (8–17). We have demonstrated that the majority of ovarian carcinoma specimens express the CSF-1 receptor, c-fms, with approximately one-half co-expressing CSF-1 (11). High levels of c-fms transcripts (measured by in situ hybridization) correlate strongly with high-grade and advanced clinical presentations prognostic of poor outcome (10, 11). CSF-1 is occasionally to rarely expressed by benign ovarian neoplasms, but prominent staining (detected by immunohistochemistry) is evident in invasive cancers (1, 15). Ovarian cancer cell lines express CSF-1 transcripts and synthesize significant quantities of biologically active CSF-1 in the medium (14, 18); such carcinoma cell production of CSF-1 contributes to elevated levels of circulating CSF-1 in the serum of ovarian cancer patients. In fact, we and others have shown that the level of CSF-1 in the serum is a sensitive and useful tumor marker in ovarian cancer patients, because it correlates with the clinical course of the disease (18–20). Recurrence or progression is frequently heralded by elevated levels of serum CSF-1 (18–20). Moreover, we have demonstrated that elevated levels of CSF-1 measured in the ascites of patients undergoing primary surgery for stages III and IV ovarian cancer is independently predictive of poor overall survival (21).

In macrophages and malignant cells of lung, breast, colon, and prostatic origin, uPA activity is strongly correlated with the ability to invade and metastasize (5, 22–30). Less is known about the role of uPA in ovarian cancer. In primary and metastatic ovarian cancer specimens, conditioned media of ovarian cancer organ cultures, and ovarian cancer cell lines, uPA is recognized to be the primary plasminogen activator (31–35). Recently, high levels of receptor-bound uPA in ovarian carcinoma specimens was found to correlate with advanced stage (36). Moreover, in two ovarian cancer cell lines,
inhibition of serine protease activity, including specifically that of uPA (37), interferes with invasion (37, 38). But despite clear evidence of CSF-1 and uPA expression in primary and metastatic ovarian cancer, no study to date has explored the effects of CSF-1 on uPA activity and invasion of ovarian cancer cells.

**MATERIALS AND METHODS**

**Cell and Cell Culture.** Both primary (Bixler, Bix2NMB, DK2NMA, Bix3) and established (SKOV3, Hey) epithelial ovarian carcinoma cells were maintained in Dulbecco’s modified Eagle’s medium—F12 Ham’s media with 1% CS supplemented with 1% penicillin-streptomycin and 10 μg/ml insulin and transferrin in T25 flasks. The primary ovarian carcinoma cell lines were previously described (11, 39, 40). Despite the recent finding that SKOV3 and Bixler share identical DNA “fingerprints” (41), we found that these cell lines can have substantially different phenotypes, as demonstrated in this manuscript. The melanoma cell line (Bowes) and the prostate cancer cell line (PC-3) was obtained from the American Type Culture Collection and were used as controls for the invasion assay. They were maintained in the same media but with 10% fetal bovine serum. Prior to RNA isolation, the cells were serum starved in the same media for 48 h (11). For invasion assays which required Bix3 cells to be stimulated with CSF-1, this 24-h stimulation was performed in serum-free media in T25 flasks prior to the invasion assays to allow for optimal measurement of CSF-1 effect on the cancer cell phenotype (11). For those experiments only, the control Bix3 cells were also changed to serum-free media for the period of the CSF-1 pretreatment (24 h). Prior to the invasion assays for all other conditions, the cells were grown in the same media with 1% NuSerum (Collaborative Research) instead of 1% CS for 24 h. All invasion assays were subsequently carried out in the presence of 1% NuSerum.

**Proteins and Antibodies.** Recombinant CSF-1 and the murine monoclonal IgG antibodies to human CSF-1 (HM 7/7.10, HM 7/2.4.4, HM 7/5.3.9.13) were kindly provided by the Immunology Department of Genetics Institute (Cambridge, MA). The two neutralizing rabbit anti-human CSF-1 polyclonal antibodies were kindly provided by E. R. Stanley (antibody raised to human urinary CSF-1; Albert Einstein, Bronx, NY), and S. Ramakrishnan (antibody raised to human recombinant CSF-1; University of Minnesota, Minneapolis, MN). HMW-uPA, human PAI-1, and the murine monoclonal IgG antibody to human PAI-1 (No. 3785) were obtained from American Diagnostica Inc. (Greenwich, CT). The murine monoclonal IgG-blocking antibody to the β chain of human uPA (No. 394) recognizes all forms of human uPA, including receptor-bound uPA, and inhibits uPA activity (American Diagnostica Inc.). The rabbit polyclonal anti-feline fms antibody (OA-11—816) was kindly provided by Cambridge Research Biochemicals Ltd. (Cheshire, United Kingdom). Synthetic fms peptide (OP-11—3816), to which the antibody was raised, was obtained from the same source. The murine monoclonal anti-human PAI-2 antibody and recombinant human PAI-2 antigen were kindly provided by U. Kralh-Matebskis (Behringwerke, Marburg, Germany). Murine IgG (Sigma) and normal rabbit serum (Vector Corp., Burlingame, CA) were used as controls in the invasion assay for the murine monoclonal and rabbit polyclonal antibodies, respectively.

**Isolation and Analysis of Total Cellular RNA.** Total cellular RNA was isolated from cells by the guanidium/cesium chloride gradient method (42). All Northern blots represented total cellular RNA extracted from cells plated on plastic. RNAs (20 μg/well) were separated by 1% agarose-formaldehyde gel electrophoresis, transferred to Gene Screen Plus (New England Nuclear, Boston, MA), and hybridized to the 32P-labeled 1.8-kilobase fragment of the coding region of human CSF-1 purified from the pXN plasmid (kindly provided by Genetics Institute, Cambridge, MA) or a 0.6-kilobase fragment of human uPA purified from pUK0321 (kindly provided by W-D. Schleuning, Schering AG, Berlin, Germany; 44). The blots were then washed to high stringency (0.1 X SSC solution at 60°C, twice each for 30 min). After autoradiographic visualization of CSF-1 transcripts, some of the blots were stripped and rehybridized to the 1.0-kilobase fragment of γ-actin purified from the pHF-1 plasmid (45) and washed to high stringency, and transcripts were visualized by autoradiography. Relative signal intensity was determined by densitometric analysis by the Bio Image Visage 2000 system. Densitometry was performed on autoradiographs exposed within the linear range of the film. Integrated absorbance over the area of each band was calculated and then normalized to that of the corresponding actin band.

**Statistical Analysis.** Pearson’s correlational analysis was carried out by the SAS statistical package (SAS Institute, Cary, NC). A P value of < 0.05 was considered significant.

**Invasion Assay.** For quantitative measurement of degree of invasion of ovarian cancer cell lines through Matrigel, the MICS developed for this purpose was used exactly as described before (46, 47), except that 0.3 ml of 1.0 mg/ml Matrigel was used to coat the 10-μm pore polycarbonate filters. When the original amount of Matrigel was used, the degree of invasion of the control cell lines (Bowes and PC-3) was in keeping with that observed by others (4). However, the amount of Matrigel was reduced in order to optimize the relative measurement of invasion of the different ovarian cancer cell lines. For instance, the degree of invasion of Bix3 cells (a weakly invasive ovarian cancer cell line) could not be discerned using the original protocol. The amount of Matrigel used in these experiments still allowed for a strict definition of invasion, because it was in excess of that used by other investigators, who examined ovarian cancer cell invasion through a Matrigel barrier (37, 38).

Briefly, these invasion studies were performed by adding 1 X 105 control or drug-treated cells to the upper chamber of the MICS chambers, after the Matrigel-coated filters were dried and rehydrated. The MICS chambers were placed in an incubator containing 5% CO2 and 95% air humidified at 37°C. The number of cells that had invaded through the Matrigel-coated filter were stained and counted after 72 h, and the percentage of invasion was calculated for each condition or cell line. When the Matrigel-coated filters were pretreated with medium containing CSF-1 and/or IgG, this was performed for 1 h at room temperature before addition of the cells. These studies of invasion through Matrigel were performed avoiding the use of inhibitors of protease action (29, 46). CS was also replaced with 1% NuSerum (Collaborative Research) to limit the protein of protease inhibitors. Moreover, strict attention was paid to keeping the passage numbers of each cell line internally consistent, and cells were always used at 70—80% confluence to minimize the SEM. No chemoattractant was used.

Matrigel, a reconstituted basement membrane matrix extracted from the murine Engelbreth-Holm-Swarm tumor (48), contains laminin, type IV collagen, heparan sulfate proteoglycan, entactin, nidogen, vitronectin, and soluble growth factors such as transforming growth factor β, basic fibroblast growth factor, and tissue-type plasminogen activator (48—50, Collaborative Research). To date neither the presence of uPA nor CSF-1 has been reported in Matrigel. Moreover, murine CSF-1 (even if present in trace amounts in Matrigel) is not able to activate human CSF-1 receptors (51, 52). Similarly, murine uPA is not able to bind to human uPA receptors (53, 54).

Invasion studies were also performed on a human simple matrix consisting of 50 μg/ml human laminin, 50 μg/ml human collagen IV in 10 mm acetic acid, and 2 mg/ml gelatin in 10 mm acetic acid (kindly provided by M. J. Hendrix and E. A. Seftor). The invasion assay was performed exactly as described above with the use of Matrigel, with strict attention paid to keeping the passage numbers and percentage of confluence of the cell lines consistent.

**ELISA for uPA, PAI-1, and PAI-2 Antigen Levels in Conditioned Media.** Serum-free conditioned media from ovarian cancer cells, in addition to serum-free conditioned media from Bix3 cells in the presence or absence of 500 ng/ml CSF-1 for 48 h, was collected for measurement of uPA antigen levels. ELISA for uPA was performed as described previously (32). Aliquots (100 μl) of conditioned media (or 0—100 ng HMW-uPA; for generation of standard curves) in fresh serum-free media were placed in a 96-well plate. The plates were incubated overnight at 4°C. After the samples were removed, 200 μl blocking solution (32) were added for 1 h at 22°C, followed by 5-min washes of 0.85% saline. Monoclonal antibody to uPA (1 μg/ml) was then added in 100 μl blocking solution, containing 1% BSA, for 4 h at 22°C, followed by another cycle of blocking and washing as above. Rabbit antihuman IgG-alkaline phosphatase conjugate (1 μg/ml) was then added in 100 μl blocking solution with 1% BSA overnight at 4°C, followed by five 5-min washes with saline. Fresh p-nitrophenyl phosphate substrate (200 μl, Sigma FAST) was added, followed by incubation at 22°C. Development of yellow color was monitored over time at 405 nm using an ELISA plate reader, with the results calculated as ng/106 cells/h.

* M. J. C. Hendrix and E. A. Seftor, personal communication.
Serum-starved conditioned media from each of the ovarian cancer cell lines for 48 h under identical conditions were concentrated 5-fold for the measurement of PAI-1 and 100-fold for PAI-2 levels (Amicon). PAI-1 and PAI-2 levels were detected by an antibody sandwich ELISA (American Diagnostica Inc.) and, after correction for the media concentration, reported as pg/ml.

Cell ELISA for the Detection of Surface-bound uPA. Bix3 cells in the logarithmic phase of growth were plated in 1% NuSerum on 96-well microtiter plates overnight and then treated with or without 250 ng/ml CSF-1 for 24 h. They were then centrifuged at the time of analysis for extent of surface-bound uPA (37). The CSF-1 treatment did not change the cell number per well. The cell ELISA for determination of uPA has been shown to detect membrane-localized uPA, since the assay was performed on growing cells in the absence of lysin or permeabilization; uPA localization to the cell surface in this assay and its binding to a surface receptor has been previously determined by indirect immunofluorescence and flow cytometry (37). Non-specific binding was measured by replacement of the primary anti-uPA antibody with an irrelevant murine IgG. The wells were washed 3 times with PBS containing 2% BSA, and surface-bound uPA was measured after 5 min HMW-uPA in binding buffer (20 mm Hepes, 0.1% BSA in PBS) or binding buffer alone was added at 37°C for 30 min to achieve saturable binding of surface receptors (37, 55, 56). ELISA for surface-bound uPA antigen was then performed as described above for conditioned media, starting with the blocking buffer and washing steps. The percentage of surface-bound uPA was calculated as the ratio of endogenous uPA detected in the absence of HMW-uPA to extent of uPA detected after the receptors were saturated with HMW-uPA. The effect of CSF-1 treatment could then be assessed on the parameter of percentage of surface-bound uPA. The effect of CSF-1 on total saturable receptors was measured after treatment with HMW-uPA, by comparing the A50±SEM in the absence or presence of CSF-1. At least 3 separate experiments were performed, with a median of 4 wells per condition.

CSF-1 Sandwich ELISA. A protocol similar to that developed by Genetics Institute was utilized, with the generous help of V. H. Van Cleave and K. Murray of Genetics Institute. Briefly, 96-well plates (Costar, Cambridge MA) were coated overnight at 4°C with the capture antibody (HM 7/2.4.4) diluted in carbonate-coating buffer to 2 μg/ml. The next day, the wells were washed 4 times in THST. The wells were then loaded with 150 μl THS with 5% gelatin and incubated for 90 min at 37°C. The wells were washed 4 times with THST. Serum-starved conditioned media from each of the ovarian cancer cell lines for 48 h under identical conditions was concentrated 100-fold (Amicon). Samples or standards (CSF-1 in PBS-1% BSA diluted in THST; 80 μl) were added per well in duplicate and incubated for 2 h at room temperature. The wells were washed again as above and 50 μl biotinylated HM 7/3.9.13 (at optimal dilution determined previously) were added to each well. The plates were incubated for 90 min at room temperature and then washed again as above. Avidin-horseradish peroxidase (50 μl Pierce) at 1:2000 in THST was added to each well for 1 h at room temperature. The plates were washed as above and 150 μl o-phenylenediamine (Pierce) substrate solution per well were added. The plates were kept at room temperature in the dark for 20 min, at which time the reaction was stopped with 6 μl 2.5 m H2SO4. The plates were read at 492 nm on a microplate reader and, after correction for media concentration, reported as pg CSF-1/ml.

Immunohistochemical Staining with Anti-Urokinase. CSF-1, fms, PAI-1, and PAI-2 Antibodies. Slides of control or CSF-1-treated ovarian cancer cells were prepared by cytospin and fixed in a 1:1 solution of acetone and methanol for 10 min, rinsed with PBS, and fixed in acetone for 5 min. The slides to be tested for uPA, fms, PAI-1, and PAI-2 antigen staining along with control slides were treated 3 times in 70 ml 10 min citrate buffer (pH 6.0) in the microwave at 700 W for 5 min. All slides were preincubated with 1.5% normal horse or rabbit serum for 1 h to block non-specific antibody binding. After a PBS wash, the slides were incubated for 1 h with a 1:25 dilution of mouse anti-human uPA IgG (No. 394), a 1:1000 dilution of mouse anti-human CSF-1 IgG (HM 7/7.7.10; Ref. 57), a 1:100 dilution of rabbit anti-feline fms antibody, a 1:25 dilution of mouse anti-human PAI-1 IgG (No. 3785), or a 1:25 dilution of mouse anti-human PAI-2 IgG for 24 h at 4°C. Negative controls were run in parallel with an irrelevant murine monoclonal IgG (Sigma) or rabbit IgG (Sigma) and with PBS in place of the primary antibody. Complete competition of binding of the anti-human uPA, CSF-1, fms, and PAI-2 antibodies with a 10 μg excess of HMW-uPA, recombinant CSF-1, synthetic fms peptide, or recombinant PAI-2, respectively, was observed, confirming the specificities of the primary antibodies. PAI-1 competition studies reflecting specificity of the PAI-1 antibody (No. 3785) have been previously described (58). After a PBS wash, the slides were incubated for 30 min with biotinylated horse anti-mouse or goat anti-rabbit antibody, washed with PBS, and then incubated for 60 min in an avidin-horseradish peroxidase complex solution (ABC Elite kit; Vector). After a further PBS wash, the slides were incubated in 0.02% hydrogen peroxide and 0.1% diaminobenzidine in 0.1 m Tris buffer (pH 7.2, Vector), washed in tap water, counterstained with hematoxylin, dehydrated in 100% ethanol, cleared in xylene, and mounted. All slides were scored by at least 2 independent readers utilizing the H score (59) with 1.0 reflecting no staining in the slide and 4.0 reflecting intense staining over the whole slide.

RESULTS

Ovarian Cancer Cell Lines Express Different Amounts of CSF-1. CSF-1 mRNA is expressed by all primary and established epithelial ovarian cancer cell lines which we have studied (40). Among 6 ovarian cancer cell lines, the degree of expression varies up to 288-fold, with Bixler being the strongest and Bix3 and DK2NMA being the weakest CSF-1 mRNA expressions (Table 1). Relative expression of CSF-1 protein levels in the conditioned media of each of these cell lines was measured by CSF-1 sandwich ELISA. Secreted CSF-1 ranged from 4.5 to 55 pg/ml, with DK2NMA expressing the least and Bixler expressing the most. CSF-1 mRNA correlated with secreted CSF-1 antigen expression with an r value of 0.79 (P = 0.06). Relative CSF-1 protein levels were also assessed among the ovarian cancer cell lines using a monoclonal antibody directed against human CSF-1, by immunohistochemistry. Among the 6 cell lines, the H score ranged from 1.0 to 3.1. While not strictly quantitative, these studies confirm that the 3 most strong CSF-1 mRNA expressions expressed the largest amounts of CSF-1 protein with an H score ≥2.4; while the rest of the cell lines had an H score ≤2.0, with Bix3 cells expressing no detectable CSF-1 antigen by immunohistochemistry.

Background characterization of the 6 ovarian cancer cell lines (Table 1) reveals that all express fms fairly strongly (immunohistochemistry) with an H score in a narrow range, from 2.95 to 4.0, with the lowest expressors being Hey and SKOV3 and the highest expressor being Bix2NMB. There was a greater range of PAI-1 expression noted in the conditioned media (ELISA), with Bix2NMB not expressing any detectable PAI-1, while Hey cells secreted 3120 pg/ml PAI-1. These findings correlated with immunohistochemical analysis of these cell lines, with the H score ranging from 1.0 to 2.0 for Bix2NMB to 3.95 for Hey cells. PAI-2 was expressed in the conditioned media to a small degree only among these 6 cell lines, ranging from 1.0 to 2.0 pg/ml (ELISA). Immunohistochemical analysis revealed a greater range of cellular PAI-2 expression, with the H score ranging from 1.4 for Bix2NMB to 3.9 for Hey cells.

Degree of Ovarian Cancer Cell Invasion of Extracellular Matrix Correlates with Endogenous CSF-1 Expression. We quantitatively measured the degree of invasion by different cell lines through a whole slide.

Table 1 Characterization of ovarian cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CSF-1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>fms&lt;sup&gt;b&lt;/sup&gt;</th>
<th>uPA&lt;sup&gt;c&lt;/sup&gt;</th>
<th>PAI-1&lt;sup&gt;d&lt;/sup&gt;</th>
<th>PAI-2&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bix3</td>
<td>1</td>
<td>3.3</td>
<td>10.5</td>
<td>130</td>
<td>3.1</td>
</tr>
<tr>
<td>DK2NMA</td>
<td>8</td>
<td>3.2</td>
<td>22.2</td>
<td>3</td>
<td>3.5</td>
</tr>
<tr>
<td>SKOV3</td>
<td>60</td>
<td>2.95</td>
<td>10.3</td>
<td>5</td>
<td>1.9</td>
</tr>
<tr>
<td>Hey</td>
<td>98</td>
<td>2.95</td>
<td>35.5</td>
<td>3120</td>
<td>3.9</td>
</tr>
<tr>
<td>Bix2NMB</td>
<td>172</td>
<td>4.0</td>
<td>1.0</td>
<td>ND&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.4</td>
</tr>
<tr>
<td>Bixler</td>
<td>288</td>
<td>3.95</td>
<td>14.0</td>
<td>9</td>
<td>3.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Relative to CSF-1 mRNA expression of Bix3 cells.
<sup>b</sup> Immunohistochemistry (H score).
<sup>c</sup> ELISA of conditioned media (ng/10<sup>6</sup> cells/h).
<sup>d</sup> ELISA of conditioned media (pg/ml).
<sup>e</sup> Not detectable.
Matrigel and show that the percentage of invasion varies from 0.50 to 10.44% among the 6 ovarian cancer cell lines (Fig. 1). The results represent the mean percentage of invasion ± SEM of at least 9 assays per cell line. Bixler, the highest CSF-1 expressor, was the most invasive cell line (10.44 ± 2.33% invasion), while only 0.90 ± 0.16% Bix3 and 0.50 ± 0.03% DK2NMA cells (the weakest CSF-1 expressors) invaded through the extracellular matrix (Fig. 1). The percentage of invasion of ovarian cancer cell lines is strongly correlated by correlational analysis with relative endogenous CSF-1 expression (r = 0.91; P = 0.01). There is a linear relationship between increasing degree of invasion and relative expression of CSF-1 mRNA. There does not appear to be a direct relationship between degree of fms, PAI-1, or PAI-2 expression (Table 1) and extent of invasion.

To demonstrate that our studies performed on Matrigel is a relevant in vitro model for human invasion and also to rule out potential confounding effects of cytokines and proteases in Matrigel, we tested the ability of Bix3 cells (a weak invader of Matrigel) and Hey cells (a strong invader) to invade a human simple matrix of laminin and type IV collagen (Fig. 2). We confirm that on human simple matrix Hey is a strongly invasive cell line (8.51 ± 0.47%), while Bix3 remains weakly invasive (0.33 ± 0.04%). The results represent at least 4 assays per condition. Therefore, we confirm our results with Matrigel, in the absence of potential effect from cytokines and proteases derived from the matrix and in the presence of human substrates.

**Effect of Exogenous CSF-1 on Invasion of Ovarian Cancer Cells.** To specifically study the effect of CSF-1 on invasion of ovarian cancer cells, the degree of invasion of Bix3 cells (low expressor of endogenous CSF-1) was studied in the presence or absence of exogenous CSF-1 (Fig. 3). The results represent the mean percentage of invasion ± SEM of at least 18 assays per condition. There was a 2-fold increase in the percentage of invasion of Bix3 cells, from 0.63 ± 0.08 to 1.25 ± 0.16% in the presence of CSF-1.

**Effect of Exogenous CSF-1 on Urokinase Expression in Ovarian Cancer Cells.** Because CSF-1 does not encode for a proteolytic enzyme capable of degrading extracellular matrix, and because of reports showing that CSF-1 augments uPA transcripts and activity in activated macrophages and receptor-bound uPA in lung carcinoma cell lines (3, 8), the effect of exogenous CSF-1 on uPA expression was studied in the ovarian cancer cell line, Bix3. Fig. 4 shows a Northern blot of stimulation of uPA mRNA expression of 2-fold by CSF-1. While CSF-1 does not increase the levels of secreted uPA antigen in the conditioned media of Bix3 cells measured by ELISA (not shown), stimulation of uPA transcripts by CSF-1 is paralleled by a dramatic increase in cellular associated uPA antigen levels. Urokinase antigen expression was determined by immunohistochemistry in cytospins of Bix3 cells in the absence or presence of 500 ng/ml CSF-1 for 48 h. The H score of 1.1 in Bix3 cells in the absence of CSF-1 was increased to 3.7 in the presence of CSF-1. Our finding that uPA immunohistochemical staining of Bix3 cells is primarily nuclear and cytoplasmic, with minimal membrane staining, is in agreement with such staining of breast and colorectal neoplasms (58, 60). To specifically measure the effect of CSF-1 on surface-bound uPA, we utilized the more sensitive and more quantitative cell ELISA method. The maximal extent of cell surface-bound uPA increased from 74% in the...
absence of CSF-1 to 100% (fully saturated) in the presence of CSF-1. This effect of CSF-1 on surface-bound uPA was preferential, because no significant effect of CSF-1 was demonstrated on amount of total saturable uPA receptors. The $A_{405}$ measured after saturation of uPA receptors by HMW-uPA was not changed (1.00 ± 0.05-fold) by CSF-1. This suggests that CSF-1-stimulated ovarian cancer cell invasion of 2-fold may be mediated collectively by an increase in uPA activity.

**CSF-1-stimulated Invasion of Ovarian Cancer Cells Is Mediated through Urokinase Activity.** In order to show that CSF-1-stimulated invasion of Bix3 cells is mediated by uPA activity, we stimulated Bix3 cells with CSF-1 and measured invasion in the presence or absence of preimmune IgG or anti-uPA antibody (Fig. 5). The results represent at least 9 assays per condition. We confirm our findings of a 2-fold stimulation of invasion by CSF-1 (seen in Fig. 3), which is not changed in the presence of preimmune IgG. The addition of anti-uPA antibody, however, completely blocks CSF-1 stimulation of invasion. There is no difference (Fig. 5) between the degree of invasion of the unstimulated Bix3 cells (0.46 ± 0.06%) and that of Bix3 cells stimulated with CSF-1 and measured in the presence of anti-uPA antibody (0.45 ± 0.04%).

The observation that baseline invasion is still measurable in Bix3 cells in the presence of anti-uPA antibody suggests that other factors play a role in mediating invasion of ovarian cancer cells, as has been suggested by others (37, 38). The possibility that antibody treatment may not completely abolish endogenous uPA activity may be a contributing factor (61, 62).

Because endogenous CSF-1 expression strongly correlates with invasiveness, we studied the effect of inhibition of endogenous CSF-1 on invasion of ovarian cancer cells by testing the effect of a saturable amount of two neutralizing polyclonal anti-human CSF-1 antibodies on invasion of Hey cells (a strong CSF-1 expressor). We could not demonstrate a specific effect of either antibody on inhibition of invasion of Hey cells which was significant when compared to normal rabbit serum. We do not believe that these studies rule out a contribution of endogenous CSF-1 to invasion, because our cells express both CSF-1 and its receptor, which allow for autocrine interactions which are not restricted to cell surface events (61, 62). To examine this further, the effect of the two anti-CSF-1-neutralizing antibodies (at the same final concentration and under similar conditions as utilized for the invasion assays) was tested on uPA mRNA and antigen expression (Fig. 6). No significant decrease in uPA expression could be detected after treatment of Hey cells with anti-CSF-1 antibodies alone, when compared to untreated Hey cells or those treated with normal rabbit serum. This suggests that, at least for some ligand/receptors, autocrine interactions are not always successfully
interrupted by the addition of exogenous antibodies, whose actions are limited to the cell surface (62).

**Endogenous Urokinase Expression Is Not Correlated with the Degree of Invasion of Ovarian Cancer Cells.** The Northern blot in Fig. 7 shows uPA mRNA expression by all 6 ovarian cancer cell lines. Hey was the strongest uPA expressor and expressed 48.8-fold more urokinase than Bix2NMB cells. The relative uPA antigen levels measured by ELISA in the conditioned media (Tables 1 and 2) strongly correlated with relative uPA transcript levels in the ovarian cancer cell lines ($r = 0.98$). Moreover, these findings were roughly confirmed by measurement of relative cellular associated uPA levels by immunohistochemistry; Hey cells expressed the most cellular associated uPA (H score = 4.0) and Bix2NMB cells expressed the least. The 3 most strong uPA expressors had an H score $\geq 3.5$, while the rest of the cell lines had lower H scores. Endogenous uPA expression, whether measured by levels of transcript, secreted antigen, or cellular associated antigen, does not correlate with degree of invasion of these ovarian cancer cell lines. When the immunohistochemistry slides were analyzed specifically for uPA membrane staining, Bixler (the most invasive cell line) had the strongest membrane staining, which may represent receptor-bound uPA. However, the moderate degree of membrane staining in DK2NMA cells did not correlate with their relative degree of invasion (the least invasive cell line). We could not demonstrate any effect of Matrigel (not shown) on uPA mRNA expression (as has been described for other cell types; 63) to explain the lack of correlation between uPA expression and invasion.

**Urokinase Expression Correlates with the Degree of Tumorigenicity in Nude Mice.** We have previously demonstrated tumorigenicity of the established ovarian cancer cell lines Hey and SKOV3 in nude mice, as well as the primary ovarian cancer cell lines Bix3 and Bixler (11, 39). These results and methods utilized to study tumorigenicity in nude mice have been previously published (11, 39). The cells were injected into the peritoneal cavity of nude mice, which represents an orthotopic location for ovarian cancer. A virulent tumor was derived from the injection of Hey cells, characterized by massive i.p. growth, which resulted in the early death (<1 month) of these animals (39). This contrasts with the slowly growing nature of tumors derived from SKOV3, Bix3, and Bixler cells. While their degree of tumorigenicity did not correlate with her2/neu (39) or CSF-1 expression, they do correlate with relative uPA mRNA and antigen expression (Table 2).

**Effect of Inhibition of Endogenous Urokinase on Invasion of Ovarian Cancer Cells.** To study the role of endogenous urokinase in invasion of ovarian cancer cells, we tested the effect of anti-urokinase antibodies on invasion of Hey cells (a strong CSF-1 and uPA expressor, one of the more invasive cell lines, and one which results in virulent tumors in nude mice). As Fig. 8 demonstrates, anti-urokinase antibodies specifically blocked invasion of Hey cells by 50% when compared to control cells or those treated with preimmune IgG. The results represent at least 9 assays per condition, and the percentage of invasion did not change when the antibody concentration was doubled (20 $\mu$g/ml anti-urokinase antibodies, not shown). These data suggest an important contribution of endogenous urokinase activity to ovarian cancer cell invasion but also support our finding (Fig. 5) that urokinase activity may not be an exclusive mediator of ovarian cancer cell invasion. This finding may also underlie the lack of direct correlation between endogenous uPA expression and invasion.

**DISCUSSION**

We studied the effect of CSF-1 on ovarian cancer cell lines in an *in vitro* model of the malignant phenotype of invasion; our results complement the *in vivo* findings that CSF-1 denotes poor prognosis in ovarian cancer patients. We found that CSF-1 stimulates invasion of ovarian cancer cells and that this augmented invasion is completely mediated by uPA activity. Co-expression of CSF-1 and uPA transcripts and protein of varying degrees was demonstrated in the 6 primary and established ovarian cancer cell lines studied. The degree of invasion of these ovarian cancer cells through a Matrigel matrix barrier (Fig. 1) strongly correlated with endogenous CSF-1 expression, with the weakest CSF-1 expressors, Bix3 and DK2NMA cells, invading the least. These findings were confirmed by our study of invasion of a weak CSF-1 expressor, Bix3, and a strong CSF-1 expressor, Hey, through human laminin and type IV collagen (Fig. 2). Treatment of Bix3 cells with exogenous CSF-1 resulted in both a 2-fold increase in invasion (Fig. 3) and a similar increase in levels of uPA transcripts (Fig. 4) and cellular associated uPA antigen. CSF-1 specifically increased the extent of surface-bound uPA on Bix3 cells, without affecting the level of total saturable receptors. This 2-fold stimulation of invasion by CSF-1 was completely blocked by anti-uPA antibodies but remained unaffected by preimmune IgG (Fig. 5).

Our findings that CSF-1 stimulates invasiveness in ovarian cancer cells are supported by studies of CSF-1-stimulated invasiveness in breast and lung carcinoma cells (64, 65). Our findings suggest that the contribution of exogenous CSF-1 to ovarian cancer cell invasion is mediated entirely by uPA activity. These are in keeping with findings in lung carcinoma cell lines, where uPA has been demonstrated to be the primary mediator of CSF-1-stimulated invasiveness (8). However, uPA was found to be responsible for 90% of the invasive capacity of lung carcinoma cell lines (8). This is not the case in ovarian cancer cell lines, where inhibition of endogenous uPA activity leads to only a 50% inhibition of invasion (Fig. 8), which did not change when the antibody concentration was doubled. Other proteases, such as the cysteine proteases and metalloproteinases, have been shown to contribute to ovarian cancer cell invasion (37, 38). Therefore, uPA is an important contributor to ovarian cancer cell invasion.

### Table 2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>uPA mRNA expression</th>
<th>uPA antigen expression</th>
<th>Virulence of tumorigenicity (11, 39)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKOV3</td>
<td>6.0</td>
<td>10.3</td>
<td>Slow growing</td>
</tr>
<tr>
<td>Bix3</td>
<td>6.3</td>
<td>10.5</td>
<td>Slow growing</td>
</tr>
<tr>
<td>Bixler</td>
<td>15.8</td>
<td>14.0</td>
<td>Slow growing</td>
</tr>
<tr>
<td>Hey</td>
<td>48.8</td>
<td>35.5</td>
<td>Virulent with early death</td>
</tr>
</tbody>
</table>

* Relative to uPA mRNA or secreted uPA antigen expression of Bix2NMB cells.

† Slow growing, disseminated widely after >60 days; virulent, massive i.p. growth, with death <30 days.
important, but not exclusive, mediator of ovarian cancer cell invasion. This could provide an explanation for our finding of the lack of direct correlation between degree of uPA expression and degree of invasion in ovarian cancer cell lines.

Urokinase expression has been associated with the ability to metastasize widely and rapidly in several other malignancies (25, 28, 30). In this study, we noted a correlation between uPA expression and the virulence of tumorigenicity of these ovarian cancer cell lines in nude mice (Table 2). However, the virulence of tumorigenicity did not correlate with the degree of invasion of ovarian cancer cell lines. Explanations for this lack of correlation include the potential effect of endogenous cytokines and proteases contained in Matrigel on degree of invasion, the possibility that human cells may not interact in a normal fashion with mouse matrix, and, most important, the absence of host factors in an in vitro model. Our findings of an association between endogenous uPA expression of ovarian cancer cell lines and degree of virulence of tumorigenicity, however, further supports uPA as an important protease in the biology of ovarian cancer.

REFERENCES


Macrophage Colony-stimulating Factor Mediates Invasion of Ovarian Cancer Cells through Urokinase

Setsuko K. Chambers, Yixun Wang, Robert E. Gertz, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/55/7/1578

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.