Ovarian cancer remains the leading cause of death from gynecologic malignancy among women in the United States (1). Although tumor-reductive surgery and taxane- and platinum-based chemotherapy regimens are effective treatments for primary disease in the majority of ovarian cancer patients, recurrence is common and often leads to death. New therapeutic agents are needed to improve survival rates and to eventually cure patients of this deadly disease. Of the emerging therapeutic strategies, those that target stromal cells in the tumor microenvironment are particularly attractive because these cells are thought to be more genetically stable than the tumor cells themselves. Moreover, targeting the host vasculature by using anti–vascular endothelial growth factor therapy has been shown to improve survival among colorectal and ovarian cancer patients.

**Background**

Interleukin-8 (IL-8) is a proangiogenic cytokine that is overexpressed in many human cancers. We investigated the clinical and biologic significance of IL-8 in ovarian carcinoma using human samples and orthotopic mouse models.

**Methods**

Tumor expression of IL-8 was assessed by immunohistochemistry among ovarian cancer patients (n = 102) with available clinical and survival data. We examined the effect of IL-8 gene silencing with small interfering RNAs incorporated into neutral liposomes (siRNA-DOPCs), alone and in combination with docetaxel, on in vivo tumor growth, angiogenesis (microvessel density), and tumor cell proliferation in mice (n = 10 per treatment group) bearing orthotopic taxane-sensitive (HeyA8 and SKOV3ip1) and taxane-resistant (SKOV3ip2.TR) ovarian tumors. All statistical tests were two-sided.

**Results**

Of the 102 cancer specimens, 43 (42%) had high IL-8 expression and 59 (58%) had low or no IL-8 expression; high IL-8 expression was associated with advanced tumor stage (P = .019), high tumor grade (P = .031), and worse survival (median survival for patients with high vs low IL-8 expression: 1.62 vs 3.79 years; P < .001). Compared with empty liposomes, IL-8 siRNA-DOPC reduced the mean tumor weight by 32% (95% confidence interval [CI] = 14% to 50%; P = .03) and 52% (95% CI = 27% to 78%; P = .03) in the HeyA8 and SKOV3ip1 mouse models, respectively. In all three mouse models, treatment with IL-8 siRNA-DOPC plus the taxane docetaxel reduced tumor growth the most compared with empty liposomes (77% to 98% reduction in tumor growth; P < .01 for all). In the HeyA8 and SKOV3ip1 models, tumors from mice treated with IL-8 siRNA-DOPC alone had lower microvessel density than tumors from mice treated with empty liposomes (HeyA8: 34% lower, 95% CI = 32% to 36% lower [P = .002]; SKOV3ip1: 39% lower, 95% CI = 34% to 44% lower [P = .007]). Compared with empty liposomes, IL-8 siRNA-DOPC plus docetaxel reduced tumor cell proliferation by 35% (95% CI = 25% to 44%; P < .001) and 38% (95% CI = 28% to 48%; P < .001) in the HeyA8 and SKOV3ip1 models, respectively.

**Conclusions**

Increased IL-8 expression is associated with poor clinical outcome in human ovarian carcinoma, and IL-8 gene silencing decreases tumor growth through antiangiogenic mechanisms.


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Interleukin-8 (IL-8) is a potent proangiogenic cytokine that is overexpressed in most human cancers, including ovarian carcinoma (6–9). IL-8 is an 8-kDa molecule that is secreted by multiple cell types, including monocytes, neutrophils, endothelial, and mesothelial cells, and tumor cells; it is responsible for recruiting neutrophils, T cells, and basophils during immune system activation (10–13). Induction of IL-8 expression is mediated primarily by the transcription factor nuclear factor kappa B (NF-κB) (14); however, the Src/signal transducer and activator of transcription 3 (Stat3) pathway may also promote IL-8 production independent of NF-κB (15). IL-8 exerts its effects by binding to the G protein–coupled receptors CXCR1 and CXCR2. Both receptors are expressed on most tumor cells as well as on endothelial cells (16,17).

The extensive effects of increased IL-8 activity on tumor pathogenesis make it a unique therapeutic target in cancer therapy. For example, IL-8 promotes tumor growth, angiogenesis, and metastasis in murine models of several cancers (14,17–20). Moreover, blocking IL-8 activity with a monoclonal antibody has been shown to decrease tumor growth in two murine cancer models (21,22).

However, there are limited data regarding the efficacy of other therapeutic approaches that target IL-8. We recently developed a novel method for highly efficient in vivo gene silencing using neutral liposome–encapsulated small interfering RNA (siRNA) (23–25). Here we examined the clinical significance of IL-8 expression in human ovarian carcinoma and tested the therapeutic efficacy of IL-8–targeted siRNA in orthotopic mouse models of ovarian cancer.

**Methods**

**Human Ovarian Cancer Specimens**

The use of clinical specimens and clinical data was approved by the Institutional Review Board for the Protection of Human Subjects at The University of Texas M. D. Anderson Cancer Center (MDACC) and at the University of Iowa. For expression analysis, 102 paraffin-embedded epithelial ovarian cancer specimens with available clinical outcome data and confirmed diagnosis by a board-certified gynecologic pathologist were obtained from the MDACC (N = 44) and University of Iowa (N = 58) tumor banks. All patients were diagnosed from 1988 to 2006 following primary cytoreductive surgery, and 92% were treated with paclitaxel- and platinum-based adjuvant chemotherapy. Slides of tumor samples were obtained for IL-8 expression analysis based on availability of tissue, as regulated by the respective tumor bank protocols. Clinical variables obtained for correlative analyses included age at diagnosis, tumor stage (International Federation of Gynecology and Obstetric staging system [26]) and grade (27), whether ascites fluid was present, the likelihood of an optimal surgical cytoreduction (all disease nodules at the completion of primary tumor cytoreduction were smaller than 1 cm), and vital status of patients relative to disease-specific survival at the time of chart review.

**Immunohistochemistry**

Immunohistochemical analysis of IL-8 (human samples and orthotopic tumor specimens [see below]), proliferating cell nuclear antigen (PCNA; orthotopic tumors), and matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9; orthotopic tumors) was conducted on 4-µm–thick formalin-fixed paraffin-embedded epithelial ovarian cancer specimens. The slides were deparaffinized and hydrated by sequential washes in xylene; 100%, 95%, and 80% ethanol; and phosphate-buffered saline (PBS). Antigen retrieval for IL-8 was performed by incubating the slides in pepsin solution (Fisher Scientific, Waltham, MA) at 37°C for 20 minutes. Antigen retrieval for PCNA was performed by heating the slides in a microwave oven (BioGenex, EZ-retriever System, San Ramon, CA) at 98°C for 10 minutes in 0.1 M citrate buffer (pH 6.0); MMP-2 and MMP-9 staining did not require antigen retrieval. Staining for the endothelial cell marker CD31 was performed on frozen orthotopic tumor specimens that were fixed by incubation in cold acetone followed by acetone:chloroform (1:1) and did not require antigen retrieval.

Following antigen retrieval or fixation, the slides were washed with PBS and incubated with 3% H₂O₂ at room temperature for 12 minutes to block endogenous peroxidase activity. Following additional PBS washes, the slides were incubated in either 5% normal horse serum and 1% normal goat serum (IL-8, PCNA, MMP-2, and CD31) or 4% fish gelatin protein block (MMP-9; Electron Microscopy Services, Hatfield, PA) diluted in PBS at room temperature for 20 minutes to block nonspecific epitopes. The slides were then incubated with a primary antibody to IL-8 (rabbit polyclonal anti-human, 1:25 dilution; Biosource, Camarillo, CA), PCNA (PC-10 mouse monoclonal IgG, 1:50 dilution; Dako,
appropriate horseradish peroxidase (HRP)–conjugated secondary antibodies, followed by incubation with an appropriate horseradish peroxidase (HRP)–conjugated secondary antibody (IL-8, CD31, MMP-2, MMP-9: Jackson Immuno, West Grove, PA; PCNA: Serotec, Raleigh, NC) for 1 hour at room temperature and detection of antibody binding with 3,3′-diaminobenzidine substrate (Phoenix Biotechnologies, Huntsville, AL). The slides were counterstained with Gill No. 3 hematoxylin (Sigma, St Louis, MO) for 15 seconds and then washed with PBS for 1 minute and mounted with Universal Mount (Research Genetics, Huntsville, AL).

All human samples were scored for staining with the IL-8 antibody in a blinded fashion by a board-certified gynecologic pathologist (MSF). Repeat staining was performed for six specimens that did not stain well initially. Scoring of IL-8 expression was based on two variables, as previously described (28)—the percentage of positively stained tumor cells and the staining intensity. For each specimen, we assigned a percentage score of 0 (0% to <5% positively stained cells), 1 (5%–50% positively stained cells), or 2 (>50% positively stained cells) and an intensity score of 0 (low or absent staining), 1 (moderate staining), or 2 (high staining) (28). The scores for the two variables were added together to obtain an overall score for each specimen. The overall scores were dichotomized into low (scores of 0–2) or high (scores of 3–4) categories for all analyses.

Cell Lines and Cultures

The derivation and source of the human epithelial ovarian cancer cell lines HeyA8 and SKOV3ip1 have been previously described (24,25). The taxane-resistant cell line SKOV3ip2.TR (a kind gift of Dr Michael Seiden, Department of Medicine, Massachusetts General Hospital, Boston, MA) was created by serial passage of human ovarian cancer SKOV3 cells in media that contained increasing concentrations of paclitaxel (29). All ovarian cancer cell lines were maintained and propagated in RPMI-1640 medium supplemented with 15% fetal bovine serum (FBS) and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). The medium for SKOV3ip2.TR cells also contained 100–150 ng/mL paclitaxel. The derivation and characterization of the two murine endothelial cell lines we used—that is, murine ovarian endothelial cells (MOEC) and murine mesentery endothelial cells (MMECs)—have been described previously (30). Both murine cell lines were maintained at 37°C in either minimal essential medium (Gemini Bioproducts) with 10% FBS (MOECs) or RPMI-1640 medium with 15% FBS (MMECs). All experiments were performed using cells grown to 60%–80% confluence, and all cell lines were routinely tested to confirm absence of Mycoplasma. We did not authenticate any of the cell lines used.

IL-8 Gene Silencing by Transfection With siRNA

The siRNA oligonucleotide used to block IL-8 expression in ovarian cancer cell lines (IL-8 mRNA target sequence: 5′-GCC AAGGAGUGCUAAGAA-3′) was purchased from Dharmacon (Lafayette, CO). A nontargeting siRNA (mRNA target sequence: 5′-UUCUCCGAACGUCCAGUUCG-3′; purchased from Qiagen, Valencia, CA) was used as a control for all in vitro and in vivo experiments. A BLAST analysis (to compare novel sequences with those of well-characterized genes; http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) confirmed that the control siRNA demonstrated no sequence homology with any known human mRNA. For in vitro transfections, HeyA8 and SKOV3ip1 cells were plated into six-well plates at 2 × 10^5 cells per well and incubated for 24 hours. The cells were then washed with PBS and incubated overnight with 5 µg siRNA (IL-8 or control) and 30 µL RNaseFect transfection reagent (Qiagen) per well in serum-containing medium at 37°C. Twenty-four hours after transfection, the medium was replaced with fresh medium. We then purified mRNA (with the use of an RNAqueous Kit [Ambion, Austin, TX], according to the manufacturer’s instructions), or collected supernatant (medium) from the cultured cells to examine IL-8 expression by reverse transcription–polymerase chain reaction (RT-PCR) or enzyme-linked immunosorbent assay (ELISA) analyses, respectively (see below).

Liposome Incorporation of siRNA

For in vivo delivery, siRNAs (IL-8 and control) were incorporated into neutral liposomes made from 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), lyophilized, and stored at −20°C as previously described (28). Before in vivo delivery, the liposome-encapsulated siRNA preparations were rehydrated with PBS to a final concentration of either 3.5 µg or 5.0 µg per 200 µL liposome suspension.

IL-8 siRNA Therapy in an Orthotopic Ovarian Cancer Model

Female athymic nude mice (NCr- nu) were purchased from the National Cancer Institute–Frederick Cancer Research and Development Center (Frederick, MD) and housed in specific pathogen-free conditions. The mice were cared for in accordance with guidelines set forth by the American Association for Accreditation for Laboratory Animal Care and the US Public Health Service Policy on Human Care and Use of Laboratory Animals. All mouse studies were approved and supervised by the MDACC Institutional Animal Care and Use Committee.

The development and characterization of the orthotopic model of advanced ovarian cancer used in these experiments have been previously described by our laboratory (24,25). Briefly, human ovarian cancer cells grown in culture were incubated with either EDTA (HeyA8) or trypsin in EDTA (SKOV3ip1 and SKOV3ip2.TR), centrifuged at 110 g at 4°C for 7 minutes, washed twice with Hank’s balanced salt solution (Gibco, Carlsbad, CA), and resuspended at a concentration of 1.25 × 10⁶ cells per mL (HeyA8) or 5 × 10⁶ cells per mL (SKOV3ip1 and SKOV3ip2.TR). Each mouse was injected intraperitoneally with 200 µL of cell suspension. Before initiating siRNA therapy in these mice, we performed an in vivo dose–response experiment to determine the optimal dose of DOPC-encapsulated IL-8 siRNA (IL-8 siRNA-DOPC) that would efficiently reduce IL-8 expression in mice bearing orthotopic HeyA8 tumors. Approximately 17 days after intraperitoneal injection with HeyA8 cells (ie, when tumors were
palpable), mice were injected intraperitoneally with a single dose of empty liposomes (baseline control; n = 3 mice) or IL-8 siRNA-DOPC or control siRNA-DOPC at either 3.5 µg or 5.0 µg per mouse (n = 9 mice per dose) in a volume of 200 µL. At 48, 96, and 144 hours after IL-8 siRNA-DOPC injection, three mice per dose were killed by cervical dislocation and their tumors were harvested and fixed in formalin for paraffin embedding or snap frozen in optimal cutting medium (Miles, Inc., Elkhart, IN) for immunohistochemical analysis. Immunohistochemical analysis of IL-8 expression in paraffin-embedded tumors was performed (as described above) to determine the optimal dose and frequency at which IL-8 expression was reduced following a single injection of IL-8 siRNA-DOPC determined by immunohistochemistry.

To test the effect of IL-8 siRNA-DOPC on tumor growth alone and in combination with docetaxel (Aventis, Bridgewater, NJ), mice (n = 10 mice per treatment group) were injected intraperitoneally with ovarian cancer cells (HeyA8 [2.5 x 10^5 cells per mouse], SKOV3ip1 [1 x 10^6 cells per mouse], or SKOV3ip2.TR [1 x 10^6 cells per mouse]) and 1 week later (ie, before tumors were palpable) were randomly assigned to receive treatment by intraperitoneal injection with empty liposomes (200 µL), control siRNA-DOPC (3.5 µg of siRNA [200-µL injection] per mouse), IL-8 siRNA-DOPC (3.5 µg per mouse), control siRNA-DOPC plus docetaxel (50 µg per mouse and HeyA8 and 35 µg per mouse for SKOV3ip1 and SKOV3ip2.TR), or IL-8 siRNA-DOPC plus docetaxel. The mice were monitored daily for signs of adverse effects (weight loss, decreased activity, decreased food or water intake) and were killed by cervical dislocation when they appeared moribund and subjected to necropsy. The timing of necropsy was determined when any mouse from either the control or a treatment group became moribund (which ever occurred first) and was approximately 27 days after HeyA8 cell injection, 38 days after SKOV3ip1 cell injection, and 56 days after SKOV3ip2.TR cell injection. Mean tumor weights, the number of tumor nodules, the pattern of disease spread, the presence and amount of ascites, and mouse body weight were recorded at necropsy. Tumors were processed as described above. The individuals who performed the necropsies were blinded to the treatment group assignments.

Characterization of Tumor Microvessel Density and Tumor Cell Proliferation Following IL-8 siRNA Therapy in an Orthotopic Mouse Model
Quantification of microvessel density and tumor cell proliferation was done as previously described (25). Briefly, images (final magnification x100; Nikon MICROPHOT-FX, 3CCD microscope [Nikon Inc., Garden City, NY] with a Sony EXwaveHAD, 3CCD camera [Sony Corporation of America, Montvale, NJ]) and Optimas Image Analysis software [Bioscan, Edmond, WA]) were taken of five random fields from each slide. Three slides were stained for either CD31 (microvessel density analysis) or PCNA (tumor cell proliferation analysis) (one slide per mouse) for all respective treatment groups. Microvessel density was calculated by determining the average number of CD31-stained vessels in each image per slide. The percentage of positive proliferating cells (number of proliferating tumor cells [ie, positive staining] divided by total tumor cells) was calculated from PCNA staining similar to CD31 analyses and compared among treatment groups.

Analysis of IL-8 Expression in HeyA8 and SKOV3ip1 Cells With RT-PCR
RT-PCR was used to examine IL-8 mRNA expression in HeyA8 and SKOV3ip1 cells following siRNA transfection. RNA was isolated 36 hours after IL-8 or control siRNA transfection of HeyA8 and SKOV3ip1 cells with the RNAqueous kit (Ambion), according to the manufacturer’s protocol, and was then transcribed into complementary DNA (cDNA). The cDNA was used for PCR amplification of IL-8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) according to the following conditions: 94°C for 2 minutes, followed by 21 cycles of 94°C for 30 seconds, 61°C for 30 seconds, and 72°C for 2 minutes, followed by 72°C for 7 minutes. The primers used for PCR amplification were IL-8 sense (5′-CTTCTTAGAAGACGAGCAAGGAAAGACAC-3’) and antisense (5′-GTTCAAGACAGCTGTCTTCATCATCAAG-3’) and GAPDH sense (5′-GAGCCACATCGTCGAC-3’) and antisense (5′-CTTCTCATGGTCCACACC-3’). We also used RT-PCR to examine the effect of IL-8 siRNA transfection of ovarian cancer cells on the expression of IL-6 and stromal cell–derived factor-1 (SDF1; also known as CXCL12). The primers used for these reactions were IL-6 sense (5′-AGCACGCACTCACTCTTTAGCAAG-3’) and antisense (5′-CCAGAAGCTTCTCTGATTA-3’) and CXCL12 sense (5′-CTACAGATGCCATGCGATTCTTTCC-3’) and antisense (5′-CCTTCTCTAACACTGTTTCAGAAG-3’). The cDNA was resolved on 2% agarose gels and quantified using Foto/Analyst Luminary software (Fotodyne, Hartland, WI). To quantify the percentage of IL-8 silencing with IL-8 siRNA, intensities of band expression were measured (Scion Image 0.4.0.3, Scion Corporation, Frederick, MD), normalized to GAPDH expression, and reported as a percent decrease from samples treated with control siRNA. All transfections were performed in duplicate and repeated twice in each cell line to confirm results.

Expression of the IL-8 receptors CXCR1 and CXCR2 in untreated human ovarian cancer cell lines (HeyA8 and SKOV3ip1) and the murine endothelial cell lines was examined with the use of RT-PCR. PCR conditions were 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes, followed by 72°C for 7 minutes. The primers used for these reactions were murine CXCR1 sense (5′-CATGACTGTTGAGGCACTATCA-3’) and antisense (5′-GCTTACACGCAACAGATGT-3’) (31), murine CXCR2 sense (5′-ATGCATTGATGGGGTAA-3’) and antisense (5′-ATCGTATGGAGGGTGA-3’) (32), murine β-actin sense (5′-TGGATACTTCTGGTTCAG-3’) and antisense (5′-TAAAAAGCAGCCTCGACTACAGTCCG-3’) (32), human CXCR1 sense (5′-GAGCCCGGATTCGATACAG-3’) and antisense (5′-GCAGACACTGCAACACACT-3’) (33), and human CXCR2 sense (5′-ATATCGGATCACTCAG-3’) and antisense (5′-GTCAGCTTTGAGGGATGCT-3’) (33).

Enzyme-Linked Immunosorbent Assay
IL-8 protein levels in the supernatant (medium) collected from HeyA8 and SKOV3ip1 cells 48 hours after IL-8 siRNA transfection were quantified by ELISA with the use of a Quantikine Immunoassay kit (R&D Systems, Minneapolis, MN), according to the manufacturer’s protocol. In addition, serum IL-8 protein levels...
in mouse sera were measured by ELISA 2 weeks after initiation of IL-8 siRNA-DOPC therapy (also collected from other four treatment arms in the HeyA8 therapy experiment described above). Whole blood was collected via the tail vein 48 hours after the previous siRNA treatment from four mice per treatment group and pooled into Microtainer vials (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 8600 g at 4°C for 2 minutes, and serum aliquots (three wells per treatment group) were analyzed by ELISA as described above.

MMP-2 and MMP-9 protein levels were examined in HeyA8 and SKOV3ip1 cancer cells following transfection with IL-8 siRNA. Twenty-four hours after transfection, the cells were washed with PBS and incubated in serum-free medium for an additional 24 hours at 37°C. The supernatants (media) were then collected, centrifuged at 110 g, and analyzed for total MMP-2 and total MMP-9 levels using Quantikine Immunoassay kits (R&D Systems).

**Immunoblot Analysis of ERK 1/2 Phosphorylation in Murine Endothelial Cells**

MMECs were serum starved overnight at 37°C and then treated with 100 ng/mL human recombinant IL-8 (eBioscience, San Diego, CA) in serum-free medium. Cell lysates were prepared at baseline (control, no treatment) and 1, 5, 10, 15, and 60 minutes after treatment with recombinant IL-8 in modified radioimmunoprecipitation assay buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% Triton, 0.5% deoxycholate) plus 25 µg/mL leupeptin, 10 µg/mL aprotinin, 2 mM EDTA, and 1 mM sodium orthovanadate. The cell lysates were centrifuged at 19 000 g for 15 minutes at 4°C, and the protein concentrations of the resulting supernatants were determined with the use of a Protein Assay kit (Bio-Rad, Hercules, CA). Proteins (35 µg lysate per lane) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% gels and transferred to nitrocellulose membrane. The membrane was incubated in 5% milk for 2 hours at room temperature to block nonspecific binding, then with a Phospho-p44/42 MAP Kinase (Thr202/Tyr204) antibody to detect phosphorylated ERK 1/2 (1:1000 dilution; Cell Signaling, Danvers, MA) overnight at 4°C followed by a HRP-conjugated sheep anti-mouse IgG antibody (1:2000 dilution; GE Healthcare UK Limited, Buckinghamshire, UK) for 2 hours at room temperature. The membranes were washed and immunoreactive proteins were detected with the use of an enhanced chemiluminescence Western Blotting Detection Kit (GE Healthcare UK Limited). As a reference, total ERK 1/2 was detected with p44/42 MAP Kinase antibody (1:1000 dilution; Cell Signaling). To confirm equal protein loading among wells, membranes were stripped and probed for total actin. All treatments were performed in duplicate and repeated in once to confirm results.

**Murine Endothelial Cell Proliferation Assay**

MMECs (2 × 10^3) were plated in each well of a 96-well plate and treated the following day with human recombinant IL-8 (eBioscience) at the following concentrations: 0, 1, 10, 50, 100, and 500 ng/mL. We determined the number of viable cells at 1, 2, 3, and 4 days after IL-8 addition by adding 50 µL of 0.15% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) to each well. After a 2-hour incubation at 37°C, the medium and MTT were removed from each well, and the remaining cells were reconstituted in 100 µL of dimethyl sulfoxide (Sigma). Viable cells were then quantified by measuring the absorbance at 570 nm using a uQuant plate reader (Bio-Tek, Winooski, VT). Proliferation assays were performed with eight replicate wells per dose and repeated once to confirm results.

**Murine Endothelial Cell Migration Assay**

Viable MMECs (cell viability determined by trypan blue exclusion) were resuspended in serum-free medium (1 × 10^5 cells per mL), and 1 mL was added to the upper wells of a membrane invasion culture system [originally described by Hendrix et al. (34)]. A 0.1% gelatin-coated membrane separated the upper wells from the lower wells, which were filled with serum-free media that lacked (control) or contained human recombinant IL-8 at 10 ng/mL, 100 ng/mL, or 500 ng/mL (eBioscience). The membrane culture system was incubated for 6 hours at 37°C. Cells that had migrated into the bottom wells were removed and collected in 0.1% EDTA, loaded onto a 3.0-µm polycarbonate filter (Osmonics, Livermore, CA) using a Minifold I Dot-Blot System (Schleicher & Schuell, Keene, NH), and fixed, stained, and counted by light microscopy, as previously described (35). The cells in 10 randomly chosen fields (<400 final magnification) were counted by two investigators (W. M. Merritt and A. K. Sood) and expressed as a percentage of the 1 × 10^5 cells that had been plated. Experiments were performed in duplicate.

**Cell Invasion Assay**

The effect of reduced IL-8 expression on tumor cell invasion was analyzed using a membrane invasion culture system as previously described (35). Briefly, HeyA8 cells were transfected with IL-8 or control siRNA. Twenty-four hours after transfection, 1 × 10^4 viable (as determined by trypan blue exclusion) cells were resuspended in serum-free medium and added to the top wells of the membrane culture system as described above. The top and bottom wells were separated by a membrane uniformly coated with a matrix consisting of human laminin (Sigma), type IV collagen (Sigma), and gelatin (ICN Biomedical, Aurora, CO). Serum-free medium or medium that contained 5% FBS was placed into the bottom wells, and the chamber was then incubated for 24 hours at 37°C. Analysis of cell invasion was performed as described above. The invasion assays were performed in duplicate and repeated once.

**Statistical Analysis**

Fisher exact test was used to examine associations between IL-8 expression in the human samples and clinical variables. Kaplan–Meier survival curves and the log-rank test were used to examine the association between tumor expression of IL-8 and patient disease-specific survival. Multivariable analysis was performed using the Cox proportional hazards model. The data for this analysis were tested (plot of differences in log of cumulative hazard rates of tested variable against time) and found to conform to proportional hazards assumptions. For animal experiments, 10 mice were assigned per treatment group. This sample size gave 80% power to detect a 50% reduction in tumor weight at a 5% level of statistical significance. Mouse and tumor weights and the number of tumor nodules for each group were compared using Student t test (for comparisons of two groups) and analysis of variance (for multiple group comparisons). For values that were not normally distributed...
(as determined by the Kolmogrov–Smirnov test), the Mann–Whitney rank-sum test was used. All statistical tests were two-sided, and P values less than .05 were considered to be statistically significant. All confidence intervals (CIs) represent the 95% level. All statistical analyses were performed using SPSS version 12 for Windows statistical software (SPSS, Inc., Chicago, IL).

Results

Characterization and Clinical Associations of IL-8 Expression in Human Epithelial Ovarian Carcinoma

We first characterized tumor expression of IL-8 in a cohort of ovarian cancer patients and examined associations between IL-8 expression and clinical and pathological variables. The median age at diagnosis of these patients was 61 years (range = 28–86 years), and the median follow-up was 1.92 years (range = 0.19 – 12.98 years). IL-8 expression was measured in 102 human ovarian epithelial cancer specimens using immunohistochemical staining. Of the 102 cancer specimens, 43 (42%) had high IL-8 expression and 59 (58%) had low or no IL-8 expression (Figure 1, A). High tumor IL-8 expression was statistically significantly associated with advanced tumor stage (P = .019) and high tumor grade (P = .031); the association between high tumor IL-8 expression and the likelihood of suboptimal primary surgical cytoreduction approached statistical significance (P = .054; Table 1). There was no association between tumor IL-8 expression and histology or the presence of ascites.

We next evaluated whether tumor expression of IL-8 was associated with patient disease-specific survival. Kaplan–Meier curves for disease-specific survival revealed that high tumor expression of IL-8 was associated with increased ovarian cancer mortality (median survival for patients with high vs low IL-8 expression: 1.62 vs 3.79 years; P < .001) (Figure 1, B). In addition, multivariable analysis revealed that high tumor IL-8 expression remained statistically significantly associated with patient survival after controlling for primary cytoreductive surgery, tumor stage and grade, histology, and presence of ascites (Table 2). As expected, advanced-stage disease (ie, stage III or IV) and the presence of ascites were associated with poor survival (hazard ratio [HR] of death from ovarian cancer = 2.35, 95% CI = 1.24 to 4.46 [P = .009] and 2.32, 95% CI = 1.12 to 4.50 [P = .02], respectively). In addition, high IL-8 expression was also statistically significantly associated with poor disease-free survival (HR of death from ovarian cancer = 3.7, 95% CI = 2.0 to 6.8; P = .001).

IL-8 siRNA-DOPC Therapy in an Orthotopic Model of Advanced Ovarian Cancer

On the basis of the association between high IL-8 expression and poor survival in patients, we sought to examine the therapeutic efficacy of siRNA-mediated silencing of endogenous IL-8 gene expression in an orthotopic mouse model. We first confirmed that the selected IL-8–targeted siRNA effectively silenced IL-8 mRNA expression in vitro (Figure 2, A and B). To confirm the specificity of the IL-8 siRNA, we examined the effects of siRNA-mediated...

![Figure 1](https://academic.oup.com/jnci/article-abstract/100/5/359/931617/359)

**Figure 1.** Interleukin-8 (IL-8) expression in human ovarian carcinoma. A) Representative images of human ovarian tumors with low and high immunohistochemical staining for IL-8. Negative control represents a sample of ovarian cancer tissue used in the current study processed for immunohistochemistry with the secondary antibody alone. Immunohistochemistry staining of cell nuclei (blue) and IL-8 (brown) are represented in photo images. B) Kaplan–Meier curves of disease-specific mortality for patients whose ovarian tumors expressed high and low levels of IL-8. The log-rank test (two-sided) was used to compare differences between groups. Survival probabilities and 95% confidence intervals (CIs) at 2 and 4 years for low IL-8 expression were 86% (95% CI = 76% to 94%) and 44% (95% CI = 24% to 64%), respectively, and for high IL-8 expression were 43% (95% CI = 28% to 59%) and 13% (95% CI = 4% to 27%), respectively.

**Table 1.** Univariate analysis of clinicopathologic variables and interleukin-8 expression

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<tr>
<th>Variable</th>
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</tr>
<tr>
<td>Ascites§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>34</td>
<td>29</td>
</tr>
<tr>
<td>Absent</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>Cytoreduction§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimal</td>
<td>36</td>
<td>17</td>
</tr>
<tr>
<td>Suboptimal</td>
<td>23</td>
<td>24</td>
</tr>
</tbody>
</table>

* IL-8 expression was dichotomized into low (overall score 0–2) and high (3–4) groups for analysis. Overall score was obtained by adding the assigned values for IL-8 staining intensity (range 0–2) and percentage of tumor cells staining for IL-8 (range 0–2).
† Fisher exact test (two-sided).
‡ Stage (International Federation of Gynecology and Obstetric staging system [26]).
§ Data missing for one patient (ascites) and two patients (cytoreduction).
IL-8 gene silencing on the expression of two other tumor-related CXC cytokines, IL-6, and SDF-1 or CXCL12. We observed no difference in IL-6 or CXCL12 mRNA expression in either HeyA8 or SKOV3ip1 cells that were transfected with IL-8 siRNA vs with control siRNA (Supplementary Figure 1, available online). Before initiating the therapy experiments, we conducted an in vivo dose–response study of IL-8 siRNA-DOPC in mice bearing orthotopic HeyA8 tumors to establish a dose that reduced IL-8 expression. Immunohistochemical analysis revealed that within 48 hours of a single injection of 3.5 µg (Figure 2, C) or 5.0 µg (data not shown) IL-8 siRNA-DOPC, tumors from treated mice were judged to have less IL-8 than tumors from control mice based on observations by investigators (MMBE, AKS) who were blinded to the treatment groups. Furthermore, 144 hours after IL-8 siRNA-DOPC injection, tumor IL-8 expression returned to baseline level (ie, the level in mice treated with empty liposomes) (Figure 2, C). On the basis of these results, we used a twice-weekly dosing with 3.5 µg IL-8 siRNA-DOPC for the subsequent experiments.

We next examined the antitumor effects of IL-8 gene silencing with IL-8 siRNA-DOPC in mice bearing orthotopic HeyA8 and SKOV3ip1 tumors. One week after tumor cell injection, mice were randomly assigned to the following treatment groups (n = 10 mice per group): empty liposomes, control siRNA-DOPC, IL-8 siRNA-DOPC, control siRNA-DOPC plus docetaxel, and IL-8 siRNA-DOPC plus docetaxel. In both tumor models, there was no statistically significant difference in the mean weight of tumors at the time of necropsy from mice treated with empty liposomes vs those from mice treated with control siRNA-DOPC (Figure 3, A and B). However, in both models, IL-8 siRNA-DOPC reduced tumor growth as assessed by tumor weight at time of necropsy compared with empty liposomes (HeyA8: 32% reduction in tumor weight, 95% CI = 14% to 50% reduction [P = .03]; SKOV3ip1: 52% reduction, 95% CI = 27% to 78% reduction [P = .03]).

Control siRNA-DOPC plus docetaxel also reduced tumor growth compared with empty liposomes in both tumor models (HeyA8: 67% reduction, 95% CI = 55% to 79% reduction, P = .001; SKOV3ip1: 84% reduction, 95% CI = 77% to 90% reduction, P = .001). In both models, treatment with IL-8 siRNA-DOPC plus docetaxel produced the greatest reduction in tumor growth compared with empty liposomes (HeyA8: 90% reduction, 95% CI = 83% to 98% reduction, P = .001; SKOV3ip1: 98% reduction, 95%

### Table 2. Multivariable analysis of associations between clinicopathologic variables and disease-specific survival in ovarian cancer patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR (95% CI)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I or II</td>
<td>1.00 (referent)</td>
<td></td>
</tr>
<tr>
<td>III or IV</td>
<td>2.35 (1.24 to 4.46)</td>
<td>.009</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>1.00 (referent)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>1.02 (0.49 to 2.09)</td>
<td>.96</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>1.00 (referent)</td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>1.72 (0.94 to 3.14)</td>
<td>.08</td>
</tr>
<tr>
<td>Ascites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>1.00 (referent)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>2.32 (1.12 to 4.50)</td>
<td>.02</td>
</tr>
<tr>
<td>Cyttoreduction</td>
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</tr>
<tr>
<td>Suboptimal</td>
<td>1.00 (referent)</td>
<td></td>
</tr>
<tr>
<td>Optimal</td>
<td>0.61 (0.34 to 1.12)</td>
<td>.12</td>
</tr>
<tr>
<td>IL-8 expression</td>
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<td></td>
</tr>
<tr>
<td>Low</td>
<td>1.00 (referent)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>3.7 (2.0 to 6.8)</td>
<td>.001</td>
</tr>
</tbody>
</table>

*HR = hazard ratio of death from ovarian cancer; CI = confidence interval; IL-8 = interleukin-8.*

† Two-sided, from Cox proportional hazards model (verified to conform to proportional hazards assumptions [see text]).
CI = 97% to 98% reduction, *P < .001). Furthermore, IL-8 siRNA-DOPC plus docetaxel was statistically significantly more effective than control siRNA plus docetaxel in reducing tumor growth (HeyA8: 70% reduction, 95% CI = 46% to 94% reduction, *P = .006; SKOV3ip1: 86% reduction, 95% CI = 82% to 89% reduction, *P = .005.

To confirm that expression of IL-8 was suppressed during this therapy experiment, we measured the circulating serum levels of IL-8 that was secreted from the orthotopic tumors. Among mice bearing HeyA8 tumors, those treated with IL-8 siRNA-DOPC had serum IL-8 levels that were lower than those treated with empty liposomes or control siRNA-DOPC (Figure 3, D). Mice treated with either IL-8 or control siRNA plus docetaxel had serum IL-8 levels that were lower than those of mice treated with empty liposomes (control siRNA + docetaxel: 57% reduction, 95% CI = 51% to 63% reduction, *P = .002). Immunohistochemical analysis revealed that the orthotopic tumors of mice treated with IL-8 siRNA-DOPC expressed less IL-8 that was secreted from the orthotopic tumors. Among mice bearing HeyA8 tumors, those treated with IL-8 siRNA-DOPC had serum IL-8 levels that were lower than those treated with empty liposomes or control siRNA-DOPC (Figure 3, D). Mice treated with either IL-8 or control siRNA plus docetaxel had serum IL-8 levels that were lower than those of mice treated with empty liposomes (control siRNA + docetaxel: 57% reduction, 95% CI = 51% to 63% reduction, *P = .002). Immunohistochemical analysis revealed that the orthotopic tumors of mice treated with IL-8 siRNA-DOPC expressed less
(subjective assessment) IL-8 than those of mice in any of the other treatment groups (Figure 3, E).

We also examined the effect of siRNA-mediated IL-8 gene silencing on the growth of orthotopic tumors derived from taxane-resistant ovarian cancer SKOV3ip2.TR cells. As expected, control siRNA-DOPC plus docetaxel did not have a statistically significant effect on tumor growth compared with empty liposomes (11% reduction, 95% CI = 44% reduction to 22% increase, P = .72) (Figure 3, C). IL-8 siRNA-DOPC alone reduced tumor growth by 47% (95% CI = 18% to 76%, P = .05). However, in this model, IL-8 siRNA plus docetaxel had the greatest effect in tumor growth inhibition compared with empty liposomes (77% reduction, 95% CI = 65% to 88% reduction, P = .004).

In each of the three tumor models, tumor incidence did not differ statistically significantly among the treatment groups (Table 3). However, in all three models, mice treated with IL-8 siRNA-DOPC had fewer tumor nodules (and less tumor burden) than mice treated with empty liposomes or control siRNA-DOPC. For example, in the SKOV3ip1 model, the mean tumor nodule count was 43 in the empty liposome group, 36 in the control siRNA-DOPC group, and 21 in the IL-8 siRNA-DOPC group (IL-8 siRNA-DOPC compared with empty liposomes: 51% reduction, 95% CI = 3% to 82%, P = .08; IL-8 siRNA-DOPC compared with control siRNA-DOPC: 42% reduction, 95% CI = 3% to 82%, P = .11). The addition of docetaxel to the IL-8 and control siRNA-DOPC groups statistically significantly reduced tumor burden in the HeyA8 and SKOV3ip1 models compared with the empty liposome group (P < .001 for both models). However, the greatest reduction in tumor burden was noted with combination treatment of IL-8 siRNA-DOPC plus docetaxel compared with control siRNA-DOPC plus docetaxel in both the HeyA8 (P = .003) and SKOV3ip1 (P = .005) models. No obvious toxic effects of any of the treatments were observed in the animals during therapy experiments as assessed by changes in behavior, feeding habits, and mobility. Furthermore, mean body weight did not differ statistically significantly among the treatment groups (data not shown), suggesting that the eating and drinking habits of the mice were not affected by the respective treatments.

### Effect of IL-8 siRNA-DOPC on Angiogenesis

Because IL-8 is a potent angiogenic factor, we also examined the effect of IL-8 gene silencing on tumor angiogenesis by staining tumors from the therapy experiments described above with an antibody against CD31 to assess tumor microvessel density. In the HeyA8 and SKOV3ip1 models, tumors from mice treated with IL-8 siRNA-DOPC had statistically significantly lower microvessel density than tumors from mice treated with empty liposomes (HeyA8: 34% reduction, 95% CI = 32% to 36% reduction, P = .002; SKOV3ip1: 39% reduction, 95% CI = 34% to 44% reduction, P = .007) (Figure 4, A). By contrast, control siRNA-DOPC plus docetaxel had minimal effects on microvessel density in both HeyA8 and SKOV3ip1 mouse models. Microvessel density was statistically significantly decreased with IL-8 siRNA-DOPC plus docetaxel compared with the empty liposomes in the HeyA8 (29% reduction, 95% CI = 27% to 32% reduction, P = .002) and SKOV3ip1 (62% reduction, 95% CI = 59% to 66% reduction, P < .001) models.

Our finding that IL-8 siRNA-mediated therapy modulated tumor angiogenesis even though the only IL-8 that was produced in these mouse models was of human origin led us to examine whether murine endothelial cells express human IL-8–responsive chemokine receptors. Murine CXCR1 and CXCR2 mRNA expression was detected in both MOECs and MMECs; however, murine CXCR1 mRNA expression appeared more pronounced in the MOECs compared with the MMECs, whereas CXCR2 was expressed at equivalent levels in both (Figure 4, B). To investigate whether human IL-8 can activate murine endothelial cells (i.e., whether mouse chemokine receptors can be activated by human IL-8), we examined phosphorylation of ERK 1/2, a downstream target of IL-8 activation, in MMECs that had been treated with human recombinant IL-8. Immunoblot analysis revealed that ERK 1/2 phosphorylation levels increased rapidly after exposure of the cells to IL-8 and remained elevated for up to 1 hour (Figure 4, C). We also used in vitro assays to assess the effect of human recombinant human IL-8 on the proliferation and migration of MMECs. There were no observable differences in murine endothelial cell proliferation with human IL-8 treatment (Figure 4, D); however, murine endothelial cell migration increased with escalating doses of human recombinant IL-8 (Figure 4, E). These findings suggest that human IL-8 produced from tumor cells in mice may in fact reduce murine endothelial cell migration and thus reduce tumor angiogenic processes as evident in our models.

### Effect of IL-8 siRNA-DOPC Therapy on the Tumor Microenvironment

IL-8 modulation of MMP expression in tumor and endothelial cells has been shown to regulate angiogenic activity (19, 33, 37). Therefore,

### Table 3. Orthotopic tumor development in mice treated with empty liposomes or siRNA-containing liposomes with or without docetaxel

<table>
<thead>
<tr>
<th>Cell line/treatment group</th>
<th>Tumor incidence†</th>
<th>Mean no. of nodules (95% CI)</th>
<th>P‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeyA8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Empty liposomes</td>
<td>10/10</td>
<td>5.5 (4.3 to 6.7)</td>
<td>referent</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>7/10</td>
<td>5.3 (3.7 to 7.0)</td>
<td>.91</td>
</tr>
<tr>
<td>IL-8 siRNA</td>
<td>10/10</td>
<td>3.4 (1.5 to 4.5)</td>
<td>.04</td>
</tr>
<tr>
<td>Control siRNA + docetaxel</td>
<td>10/10</td>
<td>3.0 (2.6 to 4.2)</td>
<td>.009</td>
</tr>
<tr>
<td>IL-8 siRNA + docetaxel</td>
<td>9/10</td>
<td>1.2 (0.9 to 1.5)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>SKOV3ip1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Empty liposomes</td>
<td>8/10</td>
<td>43.3 (25.3 to 61.2)</td>
<td>referent</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>7/10</td>
<td>36.3 (25.4 to 47.1)</td>
<td>.45</td>
</tr>
<tr>
<td>IL-8 siRNA</td>
<td>7/10</td>
<td>21.0 (6.6 to 35.4)</td>
<td>.08</td>
</tr>
<tr>
<td>Control siRNA + docetaxel</td>
<td>8/10</td>
<td>5.3 (3.9 to 6.6)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>IL-8 siRNA + docetaxel</td>
<td>9/10</td>
<td>2.2 (1.6 to 2.9)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>SKOV3ip1.2.TR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Empty liposomes</td>
<td>8/10</td>
<td>38.0 (27.3 to 48.7)</td>
<td>referent</td>
</tr>
<tr>
<td>Control siRNA</td>
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<td>49.7 (32.3 to 67.1)</td>
<td>.3</td>
</tr>
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<td>IL-8 siRNA</td>
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<td>21.9 (15.3 to 28.5)</td>
<td>.02</td>
</tr>
<tr>
<td>Control siRNA + docetaxel</td>
<td>7/10</td>
<td>3.5 (2.2 to 5.3)</td>
<td>.99</td>
</tr>
<tr>
<td>IL-8 siRNA + docetaxel</td>
<td>9/10</td>
<td>14.9 (9.1 to 20.7)</td>
<td>.002</td>
</tr>
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</table>

* IL-8 = interleukin-8; siRNA = small interfering RNA; CI = confidence interval.
† Number of mice that developed tumors/number of mice treated.
‡ Mann–Whitney rank-sum test (data not normally distributed by Kolmogrov–Smirnov test).
on the basis of the antitumor and antivascular effects of IL-8 siRNA-DOPC observed in the mouse models, we examined the effect of IL-8 siRNA-DOPC therapy on tumor expression of MMP-2 and MMP-9. Immunohistochemical staining was performed using CD31 antigen in tumors from the HeyA8 and SKOV3ip1 tumor models. Image analysis showed a significant decrease in microvessel density in tumors treated with IL-8 siRNA-DOPC compared with control siRNA-DOPC plus docetaxel (Figure 4). The decrease was statistically significant compared with the empty liposome group (IL-8-DOPC siRNA: P = .002 and IL-8 siRNA-DOPC plus docetaxel [IL-8-si + doc]: P = .002). The decrease in microvessel density was also observed in vitro using murine mesentery endothelial cells (MMECs) and murine ovarian endothelial cells (MOECs). The decrease in microvessel density was consistent with reduced expression of MMP-2 and MMP-9. However, SKOV3ip1 cells transfected with either control siRNA or IL-8 siRNA did not express detectable MMP-9.

We also examined the effect of the reduced MMP expression following IL-8 siRNA treatment on tumor cell invasion in vitro. Twenty-four hours after siRNA transfection, HeyA8 and SKOV3ip1 cells were allowed to migrate through a collagen-based matrix-coated membrane into medium that contained or lacked 5% serum. The number of cells that had migrated into the bottom wells was counted (three wells per IL-8 concentration) and expressed as a mean percentage of the total number of cells plated. The results showed a significant decrease in cell invasion in tumors treated with IL-8 siRNA-DOPC compared with control siRNA-DOPC plus docetaxel (IL-8-si + doc: P = .002). The decrease in cell invasion was statistically significant compared with the empty liposome group (IL-8-DOPC siRNA: P = .002 and IL-8 siRNA-DOPC plus docetaxel [IL-8-si + doc]: P = .002). These results suggest that the decrease in microvessel density and cell invasion was due to the reduced expression of MMP-2 and MMP-9.
Figure 5. Effect of interleukin-8 (IL-8)–targeted therapy on matrix metalloproteinase (MMP) expression and tumor cell proliferation in mouse orthotopic tumor models. A) Immunohistochemical analysis of MMP-2 and MMP-9 expression in HeyA8 tumors harvested at the completion of IL-8 siRNA-DOPC therapy with or without docetaxel. Representative images (original magnification ×100) are shown: cell nuclei (blue) and MMP-2 and MMP-9 (brown). B) Enzyme-linked immunosorbent assay of MMP-2 and MMP-9 expression in HeyA8 and SKOV3ip1 cell lines transfected for 48 hours with IL-8 siRNA. The mean percentage of MMP-2 and MMP-9 expression in IL-8 siRNA (IL-8-si) transfected cells compared with that in cells transfected with control siRNA (c-si) for assays performed in triplicate is shown. Error bars represent 95% confidence intervals. C) HeyA8 cell invasion assay. HeyA8 cells were transfected with IL-8 siRNA or control siRNA for 24 hours and then seeded (1 × 10⁵ cells) into the top wells of a membrane invasion culture system; the bottom wells contained serum-free medium (SFM) or medium containing 5% fetal bovine serum (FBS). Average percentage of cells that had invaded the membrane separating the top and bottom wells after 24 hours is shown (three wells per treatment condition). Error bars represent 95% confidence intervals. Student’s t test used to compare differences between two groups (normal distribution of data was confirmed by the Kolmogrov–Smirnov test). *P < .001 compared with control siRNA in both conditions. D) Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) to assess cell proliferation in HeyA8 and SKOV3ip1 tumors collected at completion of IL-8 siRNA-DOPC therapy (original magnification ×100). E) Quantitation of tumor cell proliferation. Immunohistochemical staining represents nonproliferating tumor cell nuclei (blue) and proliferating tumor cell nuclei (brown) in photo image. In the graph, the percentage of proliferating cells was obtained by calculating the number of proliferating tumor cells divided by total number of cells on each field. The mean percentage was calculated by examining five random fields per slide (1 slide = 1 mouse) and three slides per treatment group. Error bars represent 95% confidence intervals. Treatment arms were compared by Student’s t test (normal distribution). *P < .001 compared with the empty lipidosome group.
following day, we determined the percentage of invaded HeyA8 cells. Compared with control siRNA, IL-8 siRNA statistically significantly reduced the percentage of invasive tumor cells by 55% (95% CI = 49% to 61%, P < .001) when serum-free medium was used as the chemoattractant and by 59% (95% CI = 54% to 63%, P < .001) when serum-containing medium was used as the chemoattractant (Figure 5, C). On the basis of these findings, it is reasonable to suggest that reduction of MMP-2 and MMP-9 levels following treatment with IL-8 siRNA may contribute to reduced tumor angiogenesis and tumor growth observed in the orthotopic mouse models.

**Effect of IL-8 siRNA-DOPC on Tumor Cell Proliferation**

Finally, we examined the effect of IL-8 gene silencing on tumor cell proliferation in vivo and in vitro. Orthotopic tumors harvested from mice in the various treatment groups were stained with an antibody against PCNA (Figure 5, D and E). Compared with empty liposomes, both IL-8 siRNA-DOPC and control siRNA-DOPC plus docetaxel reduced tumor cell proliferation in the HeyA8 (IL-8 siRNA-DOPC: 17% reduction, 95% CI = 13% to 22% reduction, P < .001; control siRNA-DOPC plus docetaxel: 24% reduction, 95% CI = 20% to 28% reduction, P < .001) and SKOV3ip1 (IL-8 siRNA-DOPC: 23% reduction, 95% CI = 20% to 26% reduction, P < .001; control siRNA-DOPC plus docetaxel: 31% reduction, 95% CI = 22% to 39% reduction, P < .001) models. However, the greatest effect was observed with combination treatment of IL-8 siRNA-DOPC plus docetaxel in both HeyA8 (35% reduction, 95% CI = 25% to 44% reduction, P < .001) and SKOV3ip1 (38% reduction, 95% CI = 28% to 48% reduction, P < .001) models. In vitro, transfection of HeyA8 cells with IL-8 siRNA had no effect on tumor cell proliferation (data not shown). These findings suggest that IL-8 siRNA-DOPC-mediated changes in the tumor environment may have contributed to inhibition of tumor cell proliferation in vivo.

**Discussion**

The key findings of this study are that IL-8 overexpression in ovarian cancer was associated with decreased patient survival and was an independent prognostic factor for poor clinical outcome and that IL-8–targeted therapy with IL-8 siRNA-DOPC in combination with chemotherapy effectively reduced tumor growth in both chemotherapy-sensitive and chemotherapy-resistant ovarian cancer models. These antitumor effects were likely due to a reduction in proangiogenic factors present in the tumor microenvironment that led to decreased angiogenesis and tumor cell proliferation following silencing IL-8 expression. Our findings suggest that IL-8 may be a potential therapeutic target in ovarian cancer.

IL-8 overexpression has been reported in multiple malignancies and is frequently associated with poor clinical outcome (7). In this study, we examined a cohort of ovarian cancer patients and found that increased IL-8 expression was associated with increased disease-specific mortality. Furthermore, even after controlling for other known prognostic indicators, an increase in tumor IL-8 levels was statistically significantly associated with decreased disease-specific survival. Several smaller studies have examined the utility of using IL-8 as a diagnostic or prognostic marker in patients with ovarian cancer (8,38–42). For example, increased IL-8 expression in ovarian cyst fluid, ascites, serum, and tumor tissue from ovarian cancer patients was found to be associated with high-grade and advanced-stage cancers, as well as with decreased disease-related patient survival (8,38–42). Collectively, these data provide the rationale for targeting IL-8 as a therapeutic approach in ovarian carcinoma.

To our knowledge, however, no studies have reported targeting IL-8 as a therapeutic strategy in ovarian cancer. Therefore, we used systemic siRNA delivery, a method previously reported by our laboratory (24,25,43), to silence IL-8 expression in an orthotopic murine model of ovarian carcinoma. In our earlier studies, we showed that intraperitoneal or intravenous injection of siRNAs incorporated into DOPC liposomes were effective in silencing the expression of specific genes (Epha2 and Fak) in nude mouse models of ovarian cancer (24,25,43). In this study, IL-8 siRNA-DOPC reduced circulating and tumor levels of IL-8 in these ovarian cancer models. More important, we demonstrated that this decrease in IL-8 expression, especially when combined with taxane-based chemotherapy, led to a statistically significant reduction in orthotopic tumor growth.

The growth and spread of ovarian cancer are directly dependent on the presence of adequate vasculature (44). Xu et al. (17) reported that IL-8 overexpression was directly associated with increased tumor vascularity and tumor cell proliferation in ovarian carcinoma. In melanoma, increased IL-8 levels were associated with increased tumor angiogenesis; conversely, a reduction in tumor microvessel density occurred following treatment with an anti–IL-8 antibody (22). IL-8 has also been shown to increase tumor cell proliferation and to prolong the survival of human endothelial cells and enhance their ability to form tubules, which supports the theory that the proangiogenic effects of IL-8 are due to activation of both tumor and endothelial cells (20,22). Previous studies (23,45–47) have demonstrated the benefit of targeting ovarian cancer cells and tumor-associated endothelial cells with combination regimens that include biologic and chemotherapeutic agents. Our demonstration in this study that treatment of mice with IL-8 siRNA-DOPC in combination with docetaxel reduced microvessel density in orthotopic ovarian tumors suggests that murine endothelial cells may respond to the IL-8 secreted by the human tumor cells. Although mice do not produce IL-8, they do express murine CXCR1 and CXCR2 receptors that are 60%–70% homologous to human CXCR1 and CXCR2 (31,36,48,49). Moreover, human IL-8 has been shown to activate murine CXCR1 and CXCR2 by increasing murine neutrophil migration (36). These effects were inhibited following deletion of both CXCR1 and CXCR2 receptors (36,48). Together, these findings prompted us to investigate the effects of IL-8 produced by orthotopic tumors on murine endothelial cells in vitro. We demonstrated that MMECs and MOECs express the murine homologs of CXCR1 and CXCR2 (31,36,48,49). More important, treatment of murine endothelial cells with recombinant human IL-8 led to receptor activation (as determined by increased ERK 1/2 phosphorylation) and promotion of endothelial cell migration. Together, these data suggest that IL-8 secreted by human orthoptenic xenograft tumors may play an important role in promoting the tumor angiogenesis observed in this and possibly other preclinical murine models.
Patient survival and response to therapy are directly dependent on tumor burden and the effect on tumor growth following treatment with chemotherapeutic agents. Unfortunately, most ovarian cancer patients ultimately develop recurrent cancer and die from the disease due to the development of resistance to available therapies. Therefore, developing biologic agents that target multiple components in the tumorigenesis pathway may provide a novel approach to reduce tumor growth and spread. For example, members of the MMP family of proteins promote tumor angiogenesis as well as cellular detachment, invasion, and metastasis, and some MMP family members, including MMP-2 and MMP-9, are reported to be regulated by IL-8 expression (18,19,33,50). IL-8 induces MMP-2 and MMP-9 expression in bladder cancer and melanoma cell lines, which contributed to increased tumor cell invasion in vitro (19,21). In this study, we demonstrated that IL-8–targeted therapy reduced MMP expression in an orthotopic ovarian cancer model and decreased tumor cell invasion in vitro. Although the clinical benefit of directly inhibiting MMP activity remains unclear (51–54), the antiangiogenic and anti-invasive properties observed in this study following IL-8 silencing, which occurred in part through inhibition of MMP expression, suggest a promising role for IL-8–targeted therapy in future studies.

Targeted therapy offers a unique opportunity to inhibit the activity of specific genes that are critical for tumor growth and metastasis. In this study, we used a novel method for targeting IL-8 with siRNA. Previous investigators have attempted to use siRNA-based applications as a therapeutic strategy in preclinical investigations (55); however, ineffective delivery of the siRNAs limited tumor cell penetration and gene silencing. We have previously demonstrated that siRNA-DOPC strategies display therapeutic efficacy with no evidence of toxic or off-target (ie, nonspecific) effects (24,25,43). We have also used other strategies to target IL-8 in preclinical models of melanoma and bladder cancer, including a commercially available human IL-8 antibody (21,22,56). Indeed, antibody-based therapies have proven to be successful in clinical cancer trials (2); however, the feasibility of these approaches is limited by antibody size and the high expense of antibody production (57). In addition, the intended target may also be activated or regulated by protein–protein interactions that are independent of the antibody-binding domain (58). Another approach includes development of small-molecule inhibitors that can inhibit kinase activity in tumor cells and tumor-associated endothelial cells. However, the development of specific small-molecule inhibitors can be challenging if the target structure is not known (58). IL-8 siRNA-DOPC was highly effective in our preclinical studies and may merit clinical development.

Limitations in this study include those related to the use of cell lines and mice as a preclinical model of human malignancies. For example, cell lines may not reflect the heterogeneity seen in human tumors. Furthermore, tumors in mice may not necessarily reflect the pattern of tumor growth that occurs in humans due to differing mechanisms of host immune response. Because mice do not produce IL–8, it is possible that toxic effects of IL-8 silencing may not be fully realized in this preclinical model; such effects should be investigated further before human trials are undertaken.

In summary, we have shown that IL-8 overexpression was a poor prognostic indicator in a cohort of ovarian cancer patients and that targeted therapy with IL-8 siRNA-DOPC was effective in reducing tumor growth in both chemotheraphy-sensitive and chemotherapy-resistant models. These findings suggest a novel therapeutic approach to treat patients with ovarian cancer.

References


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