Abstract

Ovarian cancer has the highest mortality among the gynecologic malignancies. The phosphatidylinositol 3-kinase (PI3K) pathway is frequently activated, leading to increased cell survival. This study aimed to identify secreted proteins regulated by the PI3K pathway in ovarian cancer cell lines. Surface-enhanced laser desorption-ionization time-of-flight mass spectrometry with cation-exchange protein-chips was used to analyze secreted proteins from five ovarian cancer cell lines (SKOV-3, PE01, OVCAR-3, OV167, and OV207). To activate the PI3K pathway, cells were treated with 50 ng/mL epidermal growth factor (EGF) with or without 10 μM LY294002, a PI3K inhibitor. Proteins induced by EGF and inhibited by LY294002, in the m/z range 7,500 to 9,500, were purified chromatographically, identified by peptide mass fingerprinting and NH2-terminal sequencing, and confirmed by immunodepletion. Two immunologically related proteins, m/z ~8,385 and 8,922, were identified as truncated and intact forms, respectively, of interleukin 8, a chemokine previously shown to be elevated in serum of ovarian cancer patients. Another protein, m/z 7,866, was identified as CXCL1, a chemokine associated with melanoma formation and some epithelial cancers. EGF-stimulated CXCL1 levels were variably decreased by mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and p38 MAPK inhibition in the five cell lines, but only LY294002 fully reversed the EGF effect in all cell lines. Immunoreactive CXCL1 levels in 160 conditioned media were highly correlated with corresponding peak intensities at m/z 7,866 by mass spectrometry, indicating the quantitative nature of these analyses. We conclude that proteomic analysis of cell models of human disease may facilitate the discovery of pathway-dependent proteins.

Introduction

Ovarian cancer accounts for 4% of all cancers in women and has the highest mortality of all gynecologic malignancies, with the majority of women diagnosed between ages of 50 and 79 years and without a family history of this disease (1, 2). The 5-year survival rate varies from 90% for patients diagnosed at the early stage of the disease to <30% for late-stage patients. Less than a quarter of cases are diagnosed at an early stage due to the absence of a reliable diagnostic test. The most common test used clinically to assist in diagnosis, monitoring serum levels of CA125, has high sensitivity but low specificity, which leads to a high number of false positives. Because there is no effective treatment for the late stages of ovarian cancer, successful outcome relies heavily on early diagnosis (3).

In 30% to 40% of ovarian cancers, PIK3CA, an oncogene encoding the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K), is increased in copy number (4, 5), which correlates with increased activity of the PI3K signaling pathway (6). Activation of this pathway by insulin, cytokines, or growth factors can lead to increased cell survival and migration, increased cell cycle progression, and increased metastatic capability (7, 8). However, many target proteins of PI3K signaling in ovarian cancer remain unknown. Identification of these protein targets could provide an insight into signaling defects associated with ovarian cancer.

Recent advances in proteomics have provided tools for analyzing proteins in a complex biological sample. One such technology is surface-enhanced laser desorption-ionization time-of-flight mass spectrometry (SELDI-TOF MS). This technology uses protein chips with a variety of activated surfaces to isolate proteins with particular biochemical properties from a complex biological sample. In addition, it allows relative quantification of a particular protein in the mixture, making it possible to detect differentially expressed proteins.

A number of reports have identified potential tissue or serum biomarkers for ovarian, prostate, lung, and head and neck cancer using SELDI-TOF MS (9–12). Many of these studies have focused on the use of SELDI-TOF MS for identifying patterns of disease by comparing serum protein mass profiles from healthy and cancer patients. Whereas the proteomic profiling approach may prove to be of value in disease diagnosis, it does not focus on the identification of individual proteins, making it difficult to validate the biological significance of these biomarkers. Three putative biomarker identities have been reported by Zhang et al. (12), apolipoprotein A1, a form of transthyretin, and a form of inter-α-trypsin inhibitor heavy chain H4, which were differentially expressed in ovarian cancer, but these do not point to any specific cellular pathway. Because the dysregulation of a specific signaling pathway is not necessarily characteristic of a particular tumor type or stage of disease, but varies with individual tumors (13), there is a need to identify the signaling defect in each tumor to allow the possibility of specific pathway targeting in therapy. The PI3K pathway is implicated in increased cell growth and survival in many tumors, including ovarian cancers. Identification of cellular markers of PI3K signaling could therefore facilitate the development of pathway-specific therapies in tumors where this signaling pathway is active.

We now describe the use of SELDI-TOF MS to discover proteins regulated by the PI3K pathway in five ovarian cancer cell lines.
show that CXCL chemokine ligand 1 (CXCL1) and both intact and truncated interleukin 8 (IL-8) are up-regulated by PI3K signaling in ovarian cancer, and validate by immunoassay that SELDI-TOF MS provides a quantitative measure of protein concentration.

Materials and Methods

Cell lines and reagents. The human serous epithelial ovarian cancer cell lines OVCAR-3 and SKOV-3 were obtained from the American Type Culture Collection (Manassas, VA). PE01 cells, derived from a poorly differentiated ovarian adenocarcinoma, were obtained from Dr. S.P. Conover and K. Kalli (Mayo Clinic, Rochester, MN; ref. 15). Fetal bovine serum (FBS) was obtained from Thermo Trace (Melbourne, Victoria, Australia); all other tissue culture reagents were purchased from Invitrogen Australia (Mount Waverley, Victoria). Epidermal growth factor (EGF), PD98059, and SB203580 were obtained from Sigma-Aldrich (Sydney, New South Wales, Australia). The PI3K inhibitor LY294002 was from Calbiochem (San Diego, CA). Rabbit anti-human CXCL1 antibody, recombinant human CXCL1 peptide, and rabbit anti-human IL-8 antibody were obtained from Chemicon (Temecula, CA); mouse monoclonal CXCL1 antibody was from R&D Systems (Minneapolis, MN). Akt and phospho-Ser73 Akt antibodies were from Cell Signaling (Beverly, MA); mouse and rabbit immunoglobulin G (IgG) and SuperSignal West Dura enhanced chemiluminescence (ECL) reagent were from Pierce (Rockford, IL). goat anti-rabbit horseradish peroxidase–conjugated IgG, protein A beads, CM Sepharose Fast Flow, and Sephadex G-50 were obtained from Amersham Biosciences (Castle Hill, New South Wales, Australia).

Cell culture. OVCAR-3, SKOV-3, and PE01 cells were cultured in RPMI 1640 supplemented with 25 mmol/L HEPES, 10% FBS, 10 mg/mL insulin, and 0.3 mg/L glutamine. OV167 and OV207 were cultured in α-MEM medium with nucleosides supplemented with 10% FBS and 0.3 mg/L glutamine. Cultures were grown at 37°C in 5% CO2 in air. Cells were plated (5 × 105) on six-well cell culture plates and allowed to attach overnight. After attachment, culture medium was changed to serum-free medium containing 0.3 mg/L glutamine and 0.5 g/L bovine serum albumin, and duplicate wells were starved of serum for 24 hours and then subjected to one of the following treatments: (a) untreated control, (b) 50 ng/mL EGF, (c) 10 µmol/L LY294002, or (d) EGF + LY294002. After 7, 15, 24, or 30 hours, 250-µL aliquots of media were collected from all wells for SELDI-TOF analysis. Media aliquots were stored at −80°C before analysis.

To determine the contribution of different signaling pathways to CXCL1 regulation in ovarian cancer cells used in this study, cells were plated as described above. After overnight serum starvation, cells were stimulated with 50 ng/mL EGF alone or in the presence of 10 µmol/L LY294002 (PI3K inhibitor), 10 µmol/L PD98059 [mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK) kinase 1 (MEK1) inhibitor], or 10 µmol/L SB203580 (p38 MAPK inhibitor). Untreated and inhibitor-only controls were also included. Aliquots of media (200 µL) were taken from all wells at 24, 48, and 72 hours after treatment and stored at −80°C for RIA analysis.

Western blot analysis. To confirm the efficacy of the activators and inhibitors used in this study, Western blot analysis was done. Cells were plated and cultured as described above in serum-free medium for 24 hours and then underwent treatment with no addition, 50 ng/mL EGF, 50 ng/mL EGF + 10 µmol/L LY294002, or 10 µmol/L LY294002. When present, LY294002 was added 30 minutes before the test period. After 15 minutes, plates were placed on ice, cells were rinsed with 1-mL cold PBS, and scraped into 100-µL sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mmol/L DTT, 0.01% bromophenol blue]. Cell lysates were then sonicated for 15 seconds, run on 15% SDS-polyacrylamide gels with a 6% SDS-polyacrylamide stacking gel at 200 V for 45 minutes, and transferred to a nitrocellulose membrane by a wet transfer system (Bio-Rad, Hercules, CA) at 100 V for 90 minutes. After blocking with 5% milk overnight, blots were probed with either Akt phospho-Ser(Tyr) or Akt primary antibody for 1 hour at room temperature and with peroxidase-labeled secondary antibody for 1 hour at room temperature. Chemiluminescence was detected on film using ECL reagent according to the instructions of the manufacturer.

SELDI-TOF MS chip preparation. Samples were initially screened on three chip surfaces (Ciphergen, Fremont, CA): CM10 (weak anion exchange) at pH 5.0, Q10 (strong anion exchange) at pH 7.5, and IMAC-30 (immobilized metal affinity) charged with Cu2+. The CM10 surface was chosen for this study as it gave the most protein peaks. The spots were first equilibrated with 250 µL of binding buffer (100 mmol/L ammonium acetate, pH 5.0), and 100 µL of conditioned medium diluted 1:1 in binding buffer were applied to each spot using an eight-well bioprocessor (Ciphergen). Chips were then incubated with agitation (~400 rpm) for 1 hour at room temperature to allow binding of the proteins, washed with 250 µL binding buffer thrice for 5 minutes, and rinsed with deionized water twice. Spots were allowed to dry and 2 × 1 µL of matrix (50% saturated sinapinic acid in 50% acetonitrile in water containing 1% trifluoroacetic acid) were applied twice to each spot and air dried.

SELDI-TOF MS profiles. Protein profiles were obtained using a PBSilc protein chip reader (Ciphergen) and analyzed using ProteinChip Software version 3.1 (Ciphergen). Before analysis, the instrument was externally calibrated using bovine insulin (5,734.51 Da), equine cytochrome C (12,362 Da), and equine apomyoglobin (16,952.3 Da) using a linear calibration equation. High mass was set to 30,000 Da, optimizing between 5,000 and 12,000 Da. Detector sensitivity was set to 8, laser intensity to 220, and mass deflector to 3,000 Da. A total of 65 spectra were sampled from 13 positions on each spot. Two warming shots were fired at each new position at laser intensity 230 were not included in the profile. Mass accuracy was calculated from 32 spectra for the four peaks of ~7.8, 8.1, 8.3, and 8.9 kDa. The mean mass accuracy (average coefficient of variation for the four peaks) was 0.04% (3.2 mass units at m/z ±0.000).

Statistics and quality control. All time-course experiments were analyzed by repeated measures ANOVA with significance between groups determined by Fisher's protected least significant difference test. P < 0.05 was considered significant. For quality control, medium sampled from 30-hour EGF-stimulated OV167 and OV207 cells was reacted with all spots on four CM10 chips (pre-equilibrated with binding buffer) for 1 hour; spots were then washed, matrix was applied, and protein profiles were obtained as described above. Thirteen peaks were selected ranging from m/z 3,920-11,930 and their intensities were analyzed by ANOVA to calculate spot-to-spot precision. StatView version 5.0 (SAS Institute, Inc., Cary, NC) was used for these analyses.

Protein identification. Protein purification was initially done from 2 mL of conditioned medium from PE01 cells treated with 50 ng/mL EGF for 24 hours. Medium dialyzed for 24 hours against 40 volumes of 50 mmol/L ammonium acetate (pH 6.0) was applied to a CM Sepharose Fast Flow column (1 × 28 cm), equilibrated in the same buffer, at 1 mL/min. After washing with 200-mL equilibration buffer, bound protein was eluted with a linear 200-mL gradient from 0 to 0.8 mol/L NaCl. Eluted proteins were collected in 2-mL fractions and monitored on SELDI using a NP20 (normal phase) chip. Five milliliters of protein from each fraction were applied to each spot on NP20 chips, spots were dried at 37°C, matrix was applied, and profiles were obtained as described above. Fractions containing the 7.8-kDa protein were further purified using reverse-phase high-performance liquid chromatography on a 4.6 × 250-mm Jupiter 5 µ 300-A C18 column (Phenomenex, Torrance, CA), eluted with a 15% to 60% acetonitrile gradient in 0.1% trifluoroacetic acid at 1.5 mL/min. Eluted proteins were collected in 1-minute fractions in siliconized glass tubes and monitored by SELDI on NP20 chips. The fraction containing the purified 7.8-kDa protein was analyzed by tryptic peptide mass fingerprinting and NH2-terminal sequencing by the Australian Proteome Analysis Facility (University of New South Wales and Macquarie University, Sydney, New South Wales, Australia). IL-8 was purified and identified in a similar manner.

Immunodepletion. Protein identities were confirmed by immunodepletion. One hundred microliters of 30-hour EGF-stimulated medium from OV167 cells was mixed with PBS (no-antibody control), rabbit IgG (IL-8 negative control), mouse IgG (CXCL1 negative control), rabbit anti-human IL-8, or mouse monoclonal anti-human CXCL1, vortexed, and
activity was required to sustain the basal level of a protein peak intensity with LY294002 treatment would suggest that PI3K this increase was reversed by LY294002. The actual fold increase for intensity on SELDI analysis was increased at least 2-fold by EGF ered to be up-regulated by the PI3K pathway if their mean peak levels of Akt phosphorylation at Ser473, without affecting total Akt levels. OV207, OVCAR-3, and SKOV-3 ment for 15 minutes increased phosphorylated Akt in all cell lines. In all cases, treatment with LY294002 30 minutes before EGF stimulation inhibited Akt phosphorylation down to or below the basal level in the absence of EGF (Fig. 1).

**Results**

Western immunoblot analysis of phospho-Akt. EGF treatment for 15 minutes increased phosphorylated Akt in all cell lines without affecting total Akt levels. OV207, OVCAR-3, and SKOV-3 cells had high basal levels of Akt phosphorylation at Ser473, suggesting constitutive activation of PI3K signaling in these cell lines. In all cases, treatment with LY294002 30 minutes before EGF stimulation inhibited Akt phosphorylation down to or below the basal level in the absence of EGF (Fig. 1).

**SELDI analysis of conditioned media.** Proteins were considered to be up-regulated by the PI3K pathway if their mean peak intensity on SELDI analysis was increased at least 2-fold by EGF between 15 and 30 hours in at least four of the five cell lines, and this increase was reversed by LY294002. The actual fold increase for up-regulated proteins was typically 2- to 3-fold. A decrease in basal peak intensity with LY294002 treatment would suggest that PI3K activity was required to sustain the basal level of a protein represented by the peak. Spot-spot precision was determined by comparing peak intensities obtained on a pooled culture medium sample analyzed on eight spots on each of four chips. Twelve peaks were analyzed and the data binned into low (mean intensity, 1.5), medium (mean intensity, 3.2), and high (mean intensity, 14.8) peaks. The coefficient of variation of peak intensity, determined by ANOVA, was 24.8% for low peaks, 25.3% for medium peaks, and 24.8% for high peaks, indicating consistency of analytic precision over a range of peak intensities.

Five peaks with m/z values in the range of 7,500 to 9,500 were found to be up-regulated by EGF and inhibited by LY294002; of these, the three predominant peaks had mean m/z values of 7,865 ± 3.2, 8,385 ± 3.3, and 8,923 ± 3.3. Figure 2 shows typical protein mass profiles obtained from 24-hour conditioned media from all five cell lines. All five peaks were seen at some time point over the 30-hour experiment in all cell lines, with the exception of the 8.9-kDa peak which was absent from OVCAR-3 cells. Apart from this exception, basal levels of the predominant peaks (7.8, 8.3, and 8.9 kDa) each varied over a 10- to 20-fold range across the five cell lines.

**Protein purification and identification.** Media secreted by PE01 cells and OV167 cells treated with 50 ng/mL EGF for 24 hours were used for purification and identification of the 7.8- and 8.3-kDa proteins, respectively, as these cells secreted the largest amounts of these proteins. Proteins were monitored through the purification by SELDI analysis on NP20 chips. Both proteins of interest bound to a CM Sepharose column in 50 mmol/L ammonium acetate buffer at pH 6 and were eluted by a 0 to 0.8 mol/L linear NaCl gradient between 0.3 and 0.4 mol/L (Fig. 3A), determined by monitoring fractions on SELDI using NP20 chips. This is illustrated for the 7.8-kDa protein in Fig. 3B. Pooled fractions containing maximum activity (fractions 84-91 for the 7.8-kDa protein and 63-69 for the 8.3-kDa protein) were subjected to reverse-phase high-performance liquid chromatography (Fig. 3C), both proteins peaking at ~23 minutes of gradient elution (15-60% acetonitrile in 0.1% trifluoroacetic acid). Fractions were again monitored by SELDI-TOF MS on NP20 chips (Fig. 3D). The 7.8-kDa peak was identified by peptide mass fingerprinting and NH2-terminal sequencing as CXCL1 (average mass 7,866.25), also known as melanoma growth stimulatory activity (MGSA) and growth-regulated protein-α (GRO-α). The 8.3-kDa peak was identified by peptide mass fingerprinting as IL-8. By NH2-terminal sequencing, one major and one minor sequence were detected in the fraction containing the 8.3-kDa protein, which showed an additional small peak of 8.9 kDa on SELDI analysis. The major sequence, S-A-K-E-L-R-X-Q-X-L- (X = not determined), corresponds to the NH2-terminally truncated 72-residue form of mature IL-8 (IL-8[6-77]), average mass 8,386.82) whereas the minor sequence, A-V-X-P-R-S-A-K-, corresponds to the intact 77-residue form (IL-8[1-77]), average mass 8,923.49; ref. 16), consistent with the minor 8.9-kDa peak.

Based on SELDI-TOF MS peak intensities, information about the relative regulation of the intact and truncated IL-8 forms can be derived from protein mass profiles such as those shown in Fig. 2. In four of the five cell lines (SKOV-3, PE01, OV167, and OV207), IL-8 [1-77] was the predominant species, ~2- to 3-fold more abundant than IL-8[6-77]. In response to EGF stimulation, the proportion of the two forms was unchanged in OV167 and OV207 cells. In contrast, 24-hour EGF stimulation caused a relative increase in the intact/truncated IL-8 ratio in both SKOV-3 (from 1.9 ± 0.2 to 3.8 ± 0.3) and PE01 (from 3.4 ± 0.2 to 8.7 ± 2.9). As noted above, only the truncated form, IL-8[6-77], was detectable in media from OVCAR-3 cells.

**Figure 1.** Western immunoblot analysis of Akt phosphorylation in five ovarian cancer cell lines. Phosphorylation of Akt at Ser473 was measured in five ovarian cancer cell lines, as indicated, as a marker of PI3K activation. Cells were treated for 15 minutes with 50 ng/mL EGF to stimulate Akt phosphorylation in the presence or absence of 10 μmol/L LY294002 (LY) added 30 minutes before stimulation. EGF stimulated phosphorylation of Akt at Ser473 (P-Akt) in all cell lines whereas total Akt levels remained unchanged. This phosphorylation was inhibited by LY294002 down to or below the basal level in all cell lines. Representative blots from three independent experiments on each cell line.
Immunodepletion. To confirm the protein identities obtained by peptide mass fingerprinting and NH$_2$-terminal sequencing, proteins were immunoprecipitated from media of EGF-stimulated OV167 cells. Approximately 90% immunodepletion of the 7.8-kDa protein (as determined by SELDI peak intensity) was seen in the presence of anti-human CXCL1 antibody; in contrast, there was no depletion of the 7.8-kDa protein in control incubations (Fig. 4A). The 8.1-kDa peak was also depleted by >60% in the presence of anti-human CXCL1 antibody, suggesting immunologic similarity to CXCL1. No significant change in the intensities of other peaks was observed with this antibody (Fig. 4A).

Similarly, the 8.3-kDa peak was depleted by 85% to 90% in the presence of anti-human IL-8 antisera, with no effect of control incubations. Two other peaks corresponding to 8.9- and 9.1-kDa proteins were also depleted by 80% to 90% in the presence of this antibody compared with controls, suggesting their immunologic relationship to IL-8. In the case of the 9.1-kDa peak, for which no amino acid sequence was obtained due to its low abundance, the possibility also exists that it is structurally unrelated to IL-8 but interacts, and therefore coprecipitates, with it. No significant change in the intensities of other peaks was observed in any sample (Fig. 4B).

Comparison of RIA and SELDI peak intensity. Concentrations of CXCL1 in conditioned media were measured by RIA to confirm the quantitative nature of results determined by SELDI-TOF MS peak intensities. Basal 30-hour CXCL1 secretion by the five cell lines spanned a 20-fold concentration range: 9.9 ng/mL for OVCAR-3, 14.1 ng/mL for SKOV-3, 18.5 ng/mL for OV207, 20.5 ng/mL for OV167, and 208 ng/mL for PE01. Figure 5A shows that the concentration of CXCL1 in the medium of EGF treated cells increased in a time-dependent manner over the 30-hour incubation period. Concentrations of CXCL1 in the medium of untreated cells or cells treated with EGF/LY294002 or LY294002 alone remained significantly lower than in the medium of cells treated with EGF. An increase in CXCL1 concentration was first seen 7 hours after treatment in OVCA-3 cells, 15 hours after treatment in SKOV-3, PE01, and OV167 cells, and 24 hours after treatment in OV207 cells. The concentration of CXCL1 continued to increase.
beyond 24 hours in all cell lines. The relative concentration of CXCL1 was also measured using SELDI peak intensity in the media of OV167 treated with 50 ng/mL EGF over 7 to 30 hours. Media from duplicate wells were applied to the same CM10 chip to avoid chip-to-chip variation and the peak intensities for CXCL1 were measured and plotted over time (Fig. 5B). This plot also showed an increase in the CXCL1 peak intensity over time, similar to the plot of CXCL1 concentrations measured by RIA.

Concentrations of CXCL1 measured by RIA plotted against SELDI-TOF MS peak intensities show the quantitative nature of the technique (Fig. 5C). This plot of 160 data points from five cell lines shows a high correlation between the two measurements ($R^2 = 0.780$), indicating that SELDI peak intensity provides a quantitative measure of protein concentration under the test conditions.

Effect of various pathway inhibitors on CXCL1 production. Concentrations of CXCL1 in conditioned media collected from cells treated with various pathway inhibitors were measured by RIA. CXCL1 concentrations were expressed as fold difference relative to 48-hour untreated control cultures, and data were analyzed across the 72-hour time course by repeated measures ANOVA. As shown in Fig. 6, in all five cell lines, EGF significantly increased CXCL1 production ($P < 0.001$) and the increase was completely reversed by LY294002 in each case. In OVCAR3 cells, LY294002 decreased CXCL1 levels to below the control level ($P < 0.01$), indicating significant endogenous PI3K signaling in the absence of added EGF. Across the 72-hour time course, PD98059 and SB203580 caused variable inhibition of EGF-stimulated CXCL1 production in the five cell lines. PD98059 significantly attenuated the effect of EGF in OV167, OVCAR-3, and OV207 ($P < 0.01$), the reversal being essentially complete (i.e., not significantly different from control) in OV167 and OV207 whereas SB203580 had a significant effect in all cell lines, with full reversal in PE01 and OV207. Thus, in OV207 cells, all three inhibitors fully reversed EGF-stimulated production of CXCL1. This study shows that whereas the ERK or p38 MAPK pathways make a contribution to CXCL1 regulation in some of the cell lines, PI3K signaling is the common regulatory pathway for CXCL1 production in every cell line.

Discussion

In this study, we have used SELDI-TOF MS as a discovery tool to detect proteins regulated by PI3K signaling in ovarian cancer, a pathway which is frequently up-regulated in this malignancy (6). The average copy number of PIK3CA, the gene encoding the p110α catalytic subunit of PI3K, has only been reported for two of the five cell lines used in our study: 6.1 in OVCAR-3 and 3 in SKOV-3 (6). We found that these two cell lines show very similar patterns of phospho-Akt response to EGF stimulation and LY294002 inhibition, implying that PI3K signaling is similarly regulated in both cell lines.
despite differences in *PIK3CA* copy number. Furthermore, CXCL1 levels responded similarly in these cell lines to stimulation and inhibition of PI3K signaling (Fig. 6). Therefore, the *PIK3CA* copy number does not seem to have a major influence on the responsiveness of PI3K signaling in these cells.

Others have attempted to identify disease-related pathways in tumor samples using protein array technology coupled with laser capture microdissection and phospho-specific antibodies (13). The use of tumor-derived cell lines in culture offers the advantage that signaling events may be manipulated dynamically, thus aiding in the identification of pathway-specific molecules. Because mass spectrometry is not generally regarded as a quantitative technique, validation of these findings by independent methods was considered essential. To confirm that SELDI peak intensities are proportional to protein concentration, we compared immunoreactive CXCL1 levels with the intensity of the corresponding 7,866-Da peak in 160 culture medium samples. The highly significant association between these measurements validates our approach by showing the quantitative nature of SELDI-TOF MS.

CXCL1 and IL-8, both intact and truncated forms, were identified by SELDI-TOF MS and confirmed by immunodepletion studies as proteins regulated by the PI3K pathway activation in ovarian cancer cell lines. These proteins are both members of the α-chemokine family known to be involved in neutrophil degranulation and chemotaxis, as well as in cellular proliferation, and to play a part in tumor formation and angiogenesis. Both chemokines have been associated with melanoma formation (17).

Two major forms of IL-8 have been identified, 77 and 72 amino acids long, here referred to as IL-8[1-77] and IL-8[6-77], respectively (18, 19). These two forms are produced by proteolytic processing at the NH2 terminus of the protein. Both are biologically active, IL-8[1-77] being predominant in activated endothelial cells (18) and fibroblasts (20) and IL-8[6-77] being the main form secreted by T-lymphocytes (19). Previous studies have shown that IL-8[1-77] can be converted into IL-8[6-77] by proteases including plasmin

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**Figure 5.** Comparison of RIA measurement of CXCL1 and SELDI peak intensity. A, in OV167 cells, EGF at 50 ng/mL (●) significantly increased production of CXCL1 compared with control (○) as measured by RIA. This increase was most prominent between 24 and 30 hours. LY294002 at 10 μmol/L inhibited production of CXCL1 in the presence (●) or absence (○) of EGF at all tested time points. Similar results were obtained for all cell lines. B, the same samples were used to quantitate CXCL1 by the peak intensity at m/z ~ 7,860 on SELDI-TOF MS. C, correlation between SELDI peak intensity and CXCL1 concentration measured by RIA. SELDI-TOF MS peak intensity at m/z ~ 7,860 was plotted against CXCL1 concentration measured by RIA in duplicate media from all five cell lines subjected to four treatments at four time points (n = 160).

**Figure 6.** Effect of various pathway inhibitors on CXCL1 production. Cells from five ovarian cancer cell lines were treated with 50 ng/mL EGF and 10 μmol/L of the pathway inhibitors LY294002, PD98059, or SB203580 with or without EGF. Each set of eight columns represents (from left to right) control, EGF treatment, EGF + LY294002, EGF + PD98059, EGF + SB203580, LY294002 alone, PD98059 alone, and SB203580 alone. Results are expressed as fold difference in CXCL1 measured by RIA, relative to 48-hour untreated controls, and are pooled data from three independent experiments. Bars, SE. A, OV167; B, OVCAR-3; C, PE01; D, OV207; E, SKOV-3.
and thrombin (21, 22) and that the longer form is less potent at elastase release and inducing neutrophil chemotaxis (23). It is not known if there is a difference between the two forms in their proliferative and tumorigenic properties.

In this study, we detected both forms of IL-8 in four of five studied ovarian cancer cell lines, the exception being OVCAR-3, in which only IL-8[6-77] was detected. SELDI peak intensities obtained for the two forms indicate that the majority of IL-8 secreted by these cells is IL-8[1-77], which exceeds IL-8[6-77] by 2- to 3-fold. No previous reports have indicated which form of IL-8 predominates in ovarian cancer. Most previous studies of IL-8 have used immunologic techniques for its detection in ovarian cancer cells and patient body fluids. Because commercial antibodies do not differentiate between the two forms of IL-8, it is not possible to tell which form is produced by these analyses. SELDI-TOF MS, however, may prove to be a useful tool in comparing different forms of IL-8 as it separates the two isotypes by mass, allowing relative quantification of each form.

In addition to its role in the immune system, there is evidence that IL-8 plays a part in tumorigenesis. It was shown to increase proliferation of ovarian cancer cells in both cell culture and nude mice and was able to inhibit tumor necrosis factor–related apoptosis-inducing ligand–mediated apoptosis (24, 25). A number of studies reported IL-8 to be elevated in serum and cystic fluid from cancer patients compared with healthy controls and patients with benign cysts (26–29). Further, increased expression of IL-8 was correlated with higher stages of the disease (30, 31).

CXCL1, also known as melanoma growth stimulatory activity or GRO-α, was first discovered as a chemokine up-regulated in melanoma cells and has since been reported to play a role in a number of other cancers including pancreatic, lung, and breast cancer (32–34). In this study, we found that basal production rates of CXCL1 varied over a 20-fold range in five ovarian cancer cell lines although it is unknown how these rates compare to CXCL1 production by normal ovarian epithelial cells. Overexpression of CXCL1 has been reported to enhance growth and increase metastatic ability in melanocytes as well as the ability to form tumors in nude and severe combined immunodeficient mice (35).

We have shown that expression of CXCL1 is regulated by PI3K in all five studied ovarian cancer cell lines as the PI3K-specific inhibitor LY294002 reversed EGF-induced CXCL1 production in all cases. Other pathway inhibitors (i.e., the MEK inhibitor PD98059 and the p38 MAPK inhibitor SB203580) blocked the EGF effect to a variable extent across the five cell lines tested. These data indicate considerable cross-talk among different signaling pathways in individual cell lines. However, the PI3K pathway uniquely regulated CXCL1 production in all five cell lines. Interestingly, data from OV207 cells suggested involvement of all three signaling pathways in CXCL1 regulation. This cell line is histologically different from the others as it is derived from a clear cell adenocarcinoma (15). IL-8 and CXCL1 act through CXC chemokine receptor 1 (CXCR1) and CXCR2, members of the G-protein coupled receptor superfamily. Whereas IL-8 binds to both receptors, CXCL1 only binds to CXCR2. Although there are some differences in the effects of the two activated receptors, both result in calcium mobilization and activation of phospholipase C-β, which in turn leads to increases in calcium and inositol phosphates and activation of mitogen activated protein kinases (36–38). A number of studies also suggest that cross-talk exists between CXCR1/CXCR2 receptors and other signaling pathways. One such study reported that stimulation of CXCR1/CXCR2 receptors induces phosphorylation of the EGF receptor, leading to activation of the ERK1/2 pathway (39).

Overexpression of CXCR2 has been described in a number of endocrine tumors including ovarian (40) but it is unclear whether this is a frequent event in ovarian cancer.

Expression of CXCL1 is mediated by nuclear factor-κB (NF-κB) in melanoma cells, which is activated by a number of signaling pathways including the PI3K pathway (41, 42). Because NF-κB can be activated through the PI3K pathway, in addition to other mechanisms, our finding that PI3K signaling is involved in regulation of expression of CXCL1 in ovarian cancer cells suggests possible mediation by NF-κB in these cells. Other reports also suggest that an autocrine loop exists between IL-8 and CXCL1 production and NF-κB signaling. Inhibition of NF-κB in ovarian cancer cells inhibited vascular endothelial growth factor and IL-8 production and led to suppression of tumorigenicity and angiogenesis in nude mice (43).

Both IL-8 and CXCL1 are also reported to be expressed by ovarian stromal cells and granuloma-lutein cells in ovarian follicular fluid (44, 45) and by the germinal epithelium over the preovulatory follicle (46). The expression of CXCL1 and IL-8 by stromal cells was increased by treatment with human chorionic gonadotropin, suggesting possible involvement of CXCL1 and IL-8 in ovulation (47). Our findings suggest that these chemokines may be involved in cancer of the ovary as well as having a biological function in healthy ovary.

In conclusion, this study shows the application of SELDI-TOF MS to the study of tumor cell biology and emphasizes its value as a discovery tool. We detected five proteins regulated by PI3K signaling in ovarian cancer using SELDI-TOF MS and identified them by proteomic and immunologic methods as CXCL1, IL-8, and variant forms. We conclude that proteomic analysis of cell lines provides a valuable approach to identifying cellular markers of specific signaling pathway activity. Such molecules might then be further investigated for possible exploitation either as diagnostic markers or as targets for the development of pathway-specific molecular therapies.

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References


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