Peritoneal and Subperitoneal Stroma May Facilitate Regional Spread of Ovarian Cancer

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Abstract

Purpose: Epithelial ovarian cancer (EOC) is characterized by early peritoneal involvement ultimately contributing to morbidity and mortality. To study the role of the peritoneum in fostering tumor invasion, we analyzed differences between the transcriptional repertoires of peritoneal tissue lacking detectable cancer in patients with EOC versus benign gynecologic disease.

Experimental Design: Specimens were collected at laparotomy from patients with benign disease (b) or malignant (m) ovarian pathology and comprised primary ovarian tumors, paired bilateral specimens from adjacent peritoneum and attached stroma (PE), subjacent stroma (ST), peritoneal washes, ascites, and peripheral blood mononuclear cells. Specimens were immediately frozen. RNA was amplified by in vitro transcription and cohybridized with reference RNA to a custom-made 17.5k cDNA microarray.

Results: Principal component analysis and unsupervised clustering did not segregate specimens from patients with benign or malignant pathology. Class comparison identified differences between benign and malignant PE and ST specimens deemed significant by permutation test ($P_2 = 0.027$ and $P = 0.012$, respectively). A two-tailed Student's $t$ test identified 402 (bPE versus mPE) and 663 (mST versus bST) differences. After performing a permutation test, $P = 0.031$ and borderline significance for bPE versus mPE ($P = 0.056$) differences.

Conclusions: The presence of EOC may foster peritoneal implantation and growth of cancer cells by inducing factors that may represent molecular targets for disease control.

INTRODUCTION

Epithelial ovarian cancer (EOC) has a distinct predisposition to metastatic involvement of the peritoneal cavity lining. This pattern is probably linked to the presumed surface epithelium origin of EOC (1). Thus, ovarian cancer cells are anatomically placed to migrate unsupervised around the peritoneal cavity. It remains unclear, however, whether this simple anatomic advantage is solely responsible for i.p. spread of EOC or other factors may foster the implantation of migrant cancer cells onto the peritoneal lining. It has been shown, for instance, that E- and P-cadherins are progressively expressed during EOC progression and may facilitate peritoneal invasion because mesothelial cells express cadherin-binding catenins (2, 3).

Moreover, inhibition of tumor-associated proteolytic systems may represent a promising strategy to reduce the metastatic potential of solid tumors (10). Others have noted that EOC cell lines condition soluble factors might include growth factors promoting cancer cell growth by increasing adhesion, binding, and cell to cell signaling (7). Inhibition of vascular endothelial growth factor receptor leads to decreased growth of ovarian carcinoma (8). This biological effect could be enhanced by the presence in malignant ascitic fluid of proteases such as matrix metalloproteinase-9, which may promote release of vascular endothelial growth factor from mesothelial and cancer cells and increase its bioavailability (9). Moreover, inhibition of tumor-associated proteolytic systems may represent a promising strategy to reduce the metastatic potential of solid tumors (10). Others have noted that EOC cell lines condition mesothelial cells to promote adhesion and invasion through secretion of transforming growth factor β that activates plasminogen activator inhibitor type 1 (11, 12).

Similarly, the phenotype and in vitro growth pattern of mesothelial cells obtained from patients with EOC seems to be remarkably different from those obtained from non–cancer-bearing individuals (13), suggesting that a milieu exists in the peritoneal cavity capable of modulating normal cell function. Modulation might possibly occur from soluble factors present in malignant ascitic fluid or exposed to the abdominal cavity surface of patients with EOC. Soluble factors might include growth factors promoting cancer growth by increasing adhesion, binding, and cell to cell signaling (14). A recent study suggests that stromal cells associated with EOC may be genetically altered (15).

In summary, there is circumstantial evidence that the preferential invasion of the peritoneum by ovarian cancer is not exclusively due to anatomic proximity alone but also to an array of molecules secreted by cancer and other cells predisposing the peritoneum to invasion. We therefore studied the transcriptional profile of normal-appearing peritoneum and...
adherent and subjacent stroma. Specimens were obtained during laparotomy from patients with EOC or benign gynecologic conditions. Absence of cancer cell involvement of peritoneal structures was documented by histopathologic examination. Because of the limited amount of total RNA obtainable from peritoneal biopsies we adopted an amplification procedure that we have previously described and validated for gene profiling experiments (16–18). We successfully used this method to study samples obtained from fine-needle aspiration biopsies (19, 20). This analysis identified significant differences between the transcriptional profiles of patients with EOC versus benign disease suggesting that EOC may be associated with altered biology of non–cancer-bearing peritoneum and stroma; factors altered in this process may foster i.p. implantation, growth, or invasion of cancer cells contributing to the understanding of the carcinomatosis process.

**MATERIAL AND METHODS**

**Patients’ Characteristics and Sample Collection.** Specimens were obtained from patients with benign or malignant ovarian tumors during therapeutic or diagnostic laparotomy at the University of Texas M.D. Anderson Cancer Center, Houston, TX (Table 1). All patients signed an informed consent approved by the Institutional Review Board. At laparotomy, the peritoneal cavity was opened minimizing i.p. bleeding and ensuring that the sampled areas were not manipulated prior to the biopsy. Each specimen was collected and transferred to the laboratory, bisected for cDNA microarray analysis and pathology, respectively. The former was snap frozen to minimize RNA metabolism and degradation in the presence of RNAlater (Ambion Inc., Austin, TX) at −80°C and the latter in ornithine carbamyl transferase for histopathology. Specimens consisted of primary tumor samples, adjacent peritoneum and a small amount of attached stroma (PE), and subjacent stroma (ST) at the same biopsy site. Other specimens included peripheral blood mononuclear cells and peritoneum washes or ascites obtained immediately on entry into the abdominal cavity. Specimens that had microscopic tumor invasion in subsequent histopathologic examination were excluded (identified in Table 1). Additional specimens that at final pathologic assessment were not assessed as EOC were excluded from this analysis.

**RNA Preparation, Amplification, and Labeling.** Total RNA was extracted from frozen material and amplified into antisense RNA (aRNA; refs. 16, 17, 19, 20). Although the quantity of starting total RNA was in most cases sufficient for cDNA array hybridization, we have shown repeatedly that the fidelity of aRNA hybridization is at least equal and likely superior to total RNA for transcriptional profiling due to lack of contaminant ribosomal and tRNA (16, 18). Therefore, we used aRNA to increase the consistency of results particularly where low-quality total RNA was identified using Bioanalyzer 2000

<table>
<thead>
<tr>
<th>ID</th>
<th>Diagnosis</th>
<th>Stage</th>
<th>Grade</th>
<th>Side</th>
<th>Peritoneal Inflammation</th>
<th>Stroma Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>224b</td>
<td>Fibromatous nodule</td>
<td>Benign</td>
<td>NA</td>
<td>L</td>
<td>^</td>
<td>NS</td>
</tr>
<tr>
<td>225b</td>
<td>Fibrothecoma</td>
<td>Benign</td>
<td>NA</td>
<td>L</td>
<td>^</td>
<td>NS</td>
</tr>
<tr>
<td>227m</td>
<td>Endometrioid carcinoma</td>
<td>II</td>
<td>2</td>
<td>Yes</td>
<td>^</td>
<td>NS</td>
</tr>
<tr>
<td>228m</td>
<td>Papillary carcinoma</td>
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<td>3</td>
<td>BIL</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Hydrosalpinx</td>
<td>Benign</td>
<td>NA</td>
<td>R</td>
<td>^</td>
<td></td>
</tr>
<tr>
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<td>Fibrothecoma</td>
<td>Benign</td>
<td>NA</td>
<td>L</td>
<td>^</td>
<td></td>
</tr>
<tr>
<td>233m</td>
<td>Mucinous adenocarcinoma</td>
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<td>3</td>
<td>R</td>
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<td></td>
</tr>
<tr>
<td>235m</td>
<td>Mixed carcinoma</td>
<td>III</td>
<td>3</td>
<td>BIL</td>
<td></td>
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<tr>
<td>236m</td>
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<td>III</td>
<td>3</td>
<td>BIL</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>No grade</td>
<td>BIL</td>
<td></td>
<td></td>
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<tr>
<td>238b</td>
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<td>L</td>
<td>^</td>
<td></td>
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<tr>
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<td>NA</td>
<td>BIL</td>
<td>^</td>
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<tr>
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<td>Iic</td>
<td>3</td>
<td>BIL</td>
<td>^</td>
<td></td>
</tr>
<tr>
<td>243m</td>
<td>Mucinous carcinoma</td>
<td>III</td>
<td>No grade</td>
<td>BIL</td>
<td>^</td>
<td></td>
</tr>
<tr>
<td>244b</td>
<td>Fibropapillary proliferation</td>
<td>Benign</td>
<td>NA</td>
<td>L</td>
<td>^</td>
<td></td>
</tr>
<tr>
<td>245b</td>
<td>Hydrosalpinx</td>
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<td>BIL</td>
<td>^</td>
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</tr>
<tr>
<td>248m</td>
<td>Serous carcinoma</td>
<td>III</td>
<td>3</td>
<td>R</td>
<td>^</td>
<td></td>
</tr>
<tr>
<td>249m</td>
<td>Serous carcinoma</td>
<td>IIIc</td>
<td>3</td>
<td>BIL</td>
<td>^</td>
<td></td>
</tr>
<tr>
<td>250m</td>
<td>Papillary serous carcinoma</td>
<td>IIIc</td>
<td>3</td>
<td>BIL</td>
<td>^</td>
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</tr>
<tr>
<td>251b</td>
<td>Fibrothecoma</td>
<td>Benign</td>
<td>NA</td>
<td>R</td>
<td>^</td>
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</tr>
<tr>
<td>252m</td>
<td>Papillary serous carcinoma</td>
<td>Iic</td>
<td>3</td>
<td>BIL</td>
<td>^</td>
<td></td>
</tr>
<tr>
<td>253m</td>
<td>Mixed carcinoma</td>
<td>IV</td>
<td>3</td>
<td>L</td>
<td>^</td>
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<tr>
<td>254m</td>
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<td>3</td>
<td>BIL</td>
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<tr>
<td>255m</td>
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<td>1</td>
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<tr>
<td>256m</td>
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<td>3</td>
<td>BIL</td>
<td>^</td>
<td></td>
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<tr>
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<td>Serous cyst</td>
<td>Benign</td>
<td>NA</td>
<td>BIL</td>
<td>^</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Abbreviations: L, left; R, right; NA, not applicable; NS, quantity of RNA not sufficient for study; BIL, bilateral. (Yes), specimen with microscopically detectable malignant cells and not used for the study. Histopathologic evidence of inflammation: −, none; +, minimal; ++, mild; ++++, marked; ^, none by histopathologic evaluation; ^^, none by histopathologic exam or RNA for analysis; ^^^, none or too scant for histopathologic exam.

No grade, no grading is used for this histology.

*Low malignant potential.
(Agilent Technologies, Palo Alto, CA). After amplification the quality of aRNA was tested with the Agilent bioanalyzer (17). Samples from which high-quality aRNA could not be obtained were excluded. Total RNA from peripheral blood mononuclear cells pooled from six normal donors was extracted and amplified into aRNA to serve as constant reference (16, 17, 19, 20). Test and reference RNA were labeled with Cy5 (red) and Cy3 (green), respectively, and cohybridized to a custom-made 17.5k cDNA (UniGene cluster) microarray. Microarrays were printed at the Immunogenetics Section, Department of Transfusion Medicine, Clinical Center, NIH, with a configuration of 32 × 24 × 23 and contained 17,500 elements. Clones used for printing included a combination of the Research Genetics RG HsKG_031901 8k clone set and 9,000 clones selected from the RG Hs_seq_ ver 070700 40k clone set. The 17,500 spots included 12,072 uniquely named genes, 875 duplicated genes, and about 4,000 expression sequence tags. For a complete list of genes included in the Hs-CCDTM-17.5k-1px, printing is available at our Web site (http://nciarray.nci.nih.gov/gal_files/index.shtml).

**Statistical Analysis.** The raw data set was filtered according to standard procedure to exclude spots with minimum intensity arbitrarily set to an intensity parameter of <300 in both flourescence channels. If the fluorescence intensity of one channel was >300 and that of the other below <300, the fluorescence of the low-intensity channel was arbitrarily set to 300. Spots with diameters <25 μm and flagged spots were also excluded from the analysis. The filtered data were then normalized using a median ratio equivalent to 1 over the entire array. All statistical analyses were done using the log₂-based ratios normalizing the normal value in the array equal to zero. Validation and reproducibility were measured using an internal reference concordance system based on the expectation that reference concordance should perfectly collimate. The level of concordance was measured by periodically rehybridizing the same arbitrarily selected test sample (A375 melanoma cell line) with the reference sample used in all experiments (pooled peripheral blood mononuclear cells). High concordance in gene expression predicts that ratios in different experimental conditions are highly reproducible. With this goal, we analyzed seven forward and seven reciprocally labeled replicate array experiments that were hybridized periodically every other 25 cDNA array slides within each printing. This analysis showed a >95% concordance level. Nonconcordant genes were excluded from subsequent analysis (21).

Principal component analysis was done using an array analysis software (Partek Inc., St. Charles, MO) over the entire data set. Supervised class comparison utilized the BRB ArrayTool (http://linus.nci.nih.gov/BRB-ArrayTools.html) developed at the National Cancer Institute, Biometric Research Branch, Division of Cancer Treatment and Diagnosis. Unpaired samples were tested for a univariate significance threshold set at $P_2 < 0.005$. Gene clusters identified by the univariate t test were challenged with two alternative additional tests, a univariate permutation test and a global multivariate permutation test. The multivariate permutation test was calibrated to restrict the false discovery rate to 10%. Genes identified by univariate t test as differentially expressed ($P_2 < 0.005$) and a permutation test significance < 0.05 were considered truly differentially expressed. Gene function was assigned based on Database for Annotation, Visualization, and Integrated Discovery (DAVID) tool (http://apps1.niaid.nih.gov/david) and Genontology (http://linus.nci.nih.gov/BRB-Array-Tools.html).

**RESULTS**

**Patients and Samples.** Specimens were obtained from 35 patients who underwent laparotomy for benign or malignant disease of the ovary under this protocol. Statistical analysis of available specimens did not show significant difference between peritoneal washings or ascites and peripheral blood mononuclear cells derived from patients with benign or malignant conditions; therefore, these data are not discussed further. Specimens labeled peritoneum and stroma matching the requirements discussed in Materials and Methods were obtained in 26 of the 35 patients. Bilateral biopsies were obtained from the pelvic region lateral to the ovaries in areas judged to be macroscopically free of tumor and approximately 2 cm away from any visible primary tumor or peritoneal implant. Details are presented in Table 1. Ten patients had benign conditions and 16 had chemotherapy-naive EOC. Bilateral peritoneal and stromal specimens could be utilized in 15 and 16 patients, respectively, whereas in the remaining cases only one of the two biopsies yielded enough material to study. All samples underwent histopathologic analysis to check for microscopic cancer involvement cells as well as inflammation defined by the presence and intensity of the mononuclear cells infiltrate.

**Unsupervised Comparison of PE and ST Samples from Patients with Benign or Malignant Ovarian Pathology.** Transcriptional proximity was assessed by Eisen’s Pearson correlation analysis (22) and by principal component analysis (23). These analyses test whether the global gene expression pattern of individual specimens may segregate them into defined categories of particular clinical or biological significance, such as the ability to separate specimens derived from patients with benign versus malignant condition. Eisen’s correlation and clustering were done applying various filtering parameters. However, independently of the filter applied, no correlations were noted nor molecular signatures identified that could differentiate the malignant versus benign status. Principal component analysis was applied to the complete data set and similarly failed to distinguish peritoneal samples obtained from patients with benign or malignant conditions. Interestingly, among the 22 patients for which bilateral samples were available, 17 pairs clustered near each other, suggesting that the transcriptional profile of peritoneal lining is, in the majority of cases, either strongly influenced by the genetic makeup of individual patients or most likely is modulated to the same degree in different areas by the i.p. milieu.

**Supervised Analysis Comparing PE and ST Samples from Patients with Benign or Malignant Ovarian Condition.** The data set was then analyzed to test whether significant differences could be discerned in gene expression between mPE and bPE samples. Similarly, mST biopsies were compared with bST biopsies. Univariate analysis and permutation test were applied using, when available, bilateral samples from each
patient. This was done on the assumption that specimens from the same patients would be similarly conditioned by the peritoneal milieu and, therefore, would display a similar gene expression pattern. In fact, comparison of paired tissue specimens showed no significant difference. This analysis was done on 16,493 genes that passed filtering criteria (see Materials and Methods) and permutation test was set to a significance level of $P_2 \leq 0.005$ with a maximum false discovery rate of 10%. This analysis identified 402 genes to be differentially expressed between mPE and bPE and 663 between mST and bST (Table 2). Ten thousand permutations were done, confirming that the differences were significant at a 0.027 and 0.012 level, respectively. The same analysis comparing mPE and mST specimens showed no statistically significant differences, suggesting that the transcriptional changes induced by the malignant condition were at least in part overlapping. Thus, this preliminary analysis suggested that the genetic profile of peritoneal structures is strongly affected by the presence of EOC and that these effects are equally or similarly distributed within the peritoneal cavity. In addition, because no significant differences were noted between mPE and mST it is likely that gene expression alteration associated with EOC in tissue layers proximal to the peritoneal cavity are at least similar to the subjacent stromal layers.

To avoid the use of dependent samples we then validated the results by using only one PE and one ST sample per patient. The procedure reduced the number of differentially expressed genes to 236 and 327 for PE and ST samples, respectively, with a permutation test significance level borderline for PE specimens (0.056) and still significant for ST specimens (0.031; Table 2). We, therefore, focused our attention on the genes identified as differentially expressed by this most stringent analysis (the comprehensive gene list is available on request). Figure 1 shows the pattern of expression of genes differentially expressed between mPE versus bPE (Fig. 1A) and between mST versus bST (Fig. 1B). As displayed graphically, bPE and bST specimens were all quite homogeneous, suggesting that normal peritoneal samples are comparable among different individuals. In contrast, mPE and mST samples were heterogeneous, suggesting that EOC in different patients may more diversely affect the peritoneal structures. This diversity was particularly evident with ST samples of which four almost completely overlapped the genetic profile of ST specimens from patients with benign conditions. Thus, it is possible that EOC

### Table 2 Pairwise comparison based on 16,014 genes

<table>
<thead>
<tr>
<th>Class comparison</th>
<th>No. specimens</th>
<th>No. differentially expressed genes</th>
<th>Permutation significance (10,000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPE versus bPE</td>
<td>mP = 24, bP = 16</td>
<td>402</td>
<td>0.027</td>
</tr>
<tr>
<td>mST versus bST</td>
<td>mS = 26, bS = 14</td>
<td>663</td>
<td>0.012</td>
</tr>
<tr>
<td>mPE versus bPE*</td>
<td>mP = 15, bP = 10</td>
<td>236</td>
<td>0.056</td>
</tr>
<tr>
<td>mST versus bST*</td>
<td>mS = 16, bS = 8</td>
<td>327</td>
<td>0.031</td>
</tr>
<tr>
<td>mPE versus mST</td>
<td>mP = 24, mS = 26</td>
<td>87</td>
<td>0.224</td>
</tr>
</tbody>
</table>

*One sample per patient was used.
†More than one sample per patient was used when available (Table 1).
can be heterogeneous in its ability to condition surrounding structures in the peritoneal cavity, particularly those more distally situated from the peritoneal environment such as stromal structures.

Comparison of the Expression Profile of mPE with That of EOC. It is unlikely that differences between bPE and mPE were due to contamination of mPE samples by cancer cells because the specimens that contained any evidence of neoplastic infiltrate by pathologic examination were excluded from the analysis. In addition, supervised comparison of peritoneal and EOC samples using the same criteria adopted for the previous analyses identified 263 genes differentially expressed between mPE and primary tumor samples with a permutation test level of significance of 0.027, therefore precluding the possibility that the differences noted were due to the presence of malignant cells in the mPE specimens.

Functional Annotation of Genes Specific of mPE and mST. We then analyzed the annotated function of the genes identified by the previous analyses using DAVID and Genontology data analysis tools. With these tools, it was possible to stratify genes according to annotated function after exclusion of nonannotated genes such as expression sequence tags. The results of this analysis are shown in Table 3. Several genes differentially expressed between mPE and bPE had known functions associated with immune processes, tumor progression, or modulation of the extracellular matrix (Fig. 2). Additional genes differentially expressed between mST and bST with similar annotations are shown in Fig. 3. In addition, genes differentially expressed between mPE and primary tumor samples within the same functional categories are shown in Fig. 4. The annotations revealed a preferential expression by samples obtained in patients with EOC of genes with immune function, catalytic activity, involvement with adhesion and cell to cell interactions, and modulation of extracellular matrix. Overall, mPE and mST showed a more active metabolic status and extensive up-regulation of genes normally expressed during inflammatory processes compared with samples obtained from patients with benign conditions.

Genes associated with immunologic or growth regulatory function and up-regulated in mPE or mST compared with their normal counterpart were then linked according to possible relationships among one another using CellSpace knowledge miner software (see supplemental information). This software is a knowledge mining system that automatically detects, analyzes, and reports known logical relationships between molecules discussed in the biomedical literature. This analysis revealed that these genes had strong linkage to each other and were most likely associated with a chronic inflammatory status (24). Inflammatory cells were detected in 13 of 16 patients with EOC and in 2 of 9 patients with benign disease.

DISCUSSION

There is strong support for the idea that EOC development and progression is dependent on the interaction of the transforming ovarian surface epithelium and the surrounding microenvironment (2). Central to this process seem to be adhesion molecules such as E-cadherin, which is progressively expressed during neoplastic transformation and which promotes glandular formation and adherence of cancer cells to surrounding structures. In addition, E-cadherin-dependent cell-cell interactions inhibit anoikis through activation of the antiapoptotic phosphatidylinositol 3-kinase signaling and, consequently, promote survival of EOC cells shed in the peritoneal cavity (25, 26). Interestingly, transcript for β1-catenin (p120-catenin) required for proper cadherin-dependent adhesion (27) was consistently up-regulated in the peritoneal lining of patients with EOC suggesting a possible role for this molecule in fostering peritoneal implantation of EOC. β1 integrin is another molecule that becomes activated during EOC progression (2) and has been implicated in the mediation of binding of EOC cells to peritoneal mesothelium (28). In a melanoma model it was observed that β1 integrin binds to vascular cell adhesion molecule-1 under shear stress conditions (29) and this adhesion step is facilitated by chemokine receptors (30, 31). It is, therefore, interesting to note that the expression of vascular cell adhesion molecule-1 was consistently up-regulated in peritoneum specimens of EOC patients. In addition, the tight junction protein claudin 1 was found up-regulated, a finding reminiscent of the frequent overexpression of claudins in EOC (32). Furthermore, the chemokine receptor 1, which exhibits promiscuous binding to several CC chemokine ligands, was expressed. Chemokine ligands CXCL4 (PF4), CXCL6 (GCP2), CXCL8 (IL-8), CXCL14 (BRAK), and CCL13 were up-regulated in several peritoneal specimens from patients with EOC. This observation shows some similarity to studies of ascites from patients with EOC (33, 34), suggesting intense signaling among peritoneal structures during neoplastic growth controlled by a combination of adhesion molecule–dependent cell-cell interaction and by a chemokine/chemokine receptor network. Overall, the peritoneum of patients with EOC was characterized by extreme “stickiness” provided also by a large number of coagulation factors, complement components, and scavenger receptors such as CD163 and sialoadhesin. It is possible that

Table 3 Overview of genes up-regulated between various combinations of tissues categorized according to established annotations

<table>
<thead>
<tr>
<th>Relative gene expression</th>
<th>GF</th>
<th>Apo</th>
<th>Angio</th>
<th>Imm</th>
<th>Met</th>
<th>CA</th>
<th>Ad</th>
<th>Bin</th>
<th>EM</th>
<th>Sig</th>
<th>Trans</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPE/bPE</td>
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<td>1</td>
<td>3</td>
<td>23</td>
<td>4</td>
<td>33</td>
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<td>7</td>
<td>14</td>
<td>4</td>
<td>210</td>
</tr>
<tr>
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<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
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<td>4</td>
<td>2</td>
<td>27</td>
<td>2</td>
<td>43</td>
<td>10</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

NOTE: Gene function was assigned based on DAVID tool and Genontology (see text). Abbreviations: GF, growth factors; Apo, apoptosis; Angio, angiogenesis; Imm, immunology; Met, metastasization; CA, catalytic activity; Ad, adhesion; Bin, binding; EM, extracellular matrix; Sig, signaling; Trans, transcriptional regulation.
factors induced by the peritoneal lining such as tumor necrosis factor-4 may be responsible for activation of matrix proteolysis and other inflammatory reactions (35). Most likely, the transcriptional changes observed in the peritoneum and subjacent stroma of EOC patients may result from the effects of soluble factors released by the malignant tissues into the peritoneal cavity because molecules the size of chemokines and most cytokines can easily penetrate the mesothelium. For instance, the proinflammatory cytokine interleukin 6, frequently overexpressed in ascites of EOC patients (36–38) could induce many of the changes observed.

Particularly interesting is the genetic profile of subjacent stroma specimens in patients with EOC in which an intense inflammatory gene activity was observed centered predominantly on the induction of CXCL6 and CXCL8. We have previously noted that interleukin 8 (CXCL8) is detectable in a subset of melanoma metastases and when present it is co-coordinately expressed with several proinflammatory cytokines, growth factors, metalloproteinases, and IFN responsive elements, some of them also noted in this study (39). CellSpace analysis suggested a central stage for interleukin 8 in the genetic profile of peritoneal structures in patients with EOC, particularly regarding the induction of platelet factor 4 (CXCL4) whose levels have been previously found to be increased in the ascites of patients with EOC (40) and may play a role in platelet activation and induction of various coagulation factors. CXCL4 is primarily chemotactic for fibroblasts. Both CXCL4 and CXCL8 can bind to matrix proteoglycans such as decorin 6-chondroitin sulfate, which is coexpressed with myofibroblasts in EOC stroma (41), and this may facilitate the accumulation of such chemokines. In addition, platelet-derived growth factor α and β and associated receptors were found up-regulated in stromal tissues. These molecules may stimulate angiogenesis by bringing pericytes within stromal structures and induce the expression of vascular endothelial growth factor to foster cancer-cell survival after implantation (42). Platelet-derived growth factor receptors seem to play a central role in this process and have been previously considered as promising therapeutic targets against solid tumors (43). The association of the expression of platelet-derived growth factor and its α but not β receptor has been already described in EOC and is associated with poorer clinical outcome (44). Interestingly, in the peritoneal structures such combinations of molecules are present even at sites in which tumor cell implants are not detected, suggesting that other factors may influence the expression of such molecules by cells other than cancer cells (45).

Of particular interest was the up-regulation of expression of collagenase 3 (metalloproteinase-13) in stromal structures most likely induced by interleukin 8 (46), which is a potent modulator of the extracellular matrix and may play a role in cancer cell invasiveness after implantation. Metalloproteinase-13 has been already observed in the ascites of patients with advanced EOC (47), and its interstitial expression has been considered a poor prognostic marker (48). Also interesting was the expression of
carcinoembryonic antigen–related cell adhesion molecules that may be important for the adhesion of cancer cells to the extracellular matrix and at the same time through cross-linking with integrin-β3 and integrin-β7 (also up-regulated in the stromal structures) may contribute to the invasive potential of cancer cells (49). Integrin signaling is complex and may involve other receptors (e.g., growth factors; ref. 50). Thus, whereas the superficial peritoneum of patients with EOC seems to be abundant in receptors for cancer cells that may facilitate adherence of cancer cells, the deeper stromal layer is rich in molecules that promote remodeling of and adherence to the extracellular matrix together with induction of angiogenesis and other signals that foster cancer cell survival after implantation. In addition, various genes that play an important role in EOC cell growth were expressed in ST (e.g., insulin-like growth factor receptors; ref. 51) but their potential role in peritoneal structures remains unclear.

The transcriptional profile of PE and ST samples in patients with EOC is reminiscent of information derived from the renal dialysis literature describing a fibrosis syndrome induced by a chronic inflammation of peritoneal structures (52–55). In these cases and in experimental models, the peritoneal stroma first becomes thickened and fibrotic; an infiltration with macrophages then ensues that further augments the process. We have previously shown that myofibroblasts co-migrate in the stroma of EOC tissues with expression of the proteoglycans such as decorin (41). The genes identified by this study (i.e., collagens, fibroblast growth factors, platelet-derived growth factors, vascular cell adhesion molecule-1, etc.) support a profibrotic environment similar to that described in the early stages of dialysis-related fibrosis that may increase the stickiness of the peritoneal structures and, consequently, favor cancer cell implantation. Moreover, retroperitoneal fibrosis associated with coalescing fibrotic plaques are commonly seen in EOC patients with advancing disease (56), contributing to morbidity in this disease.

In summary, this is the first direct demonstration that the peritoneal lining of patients bearing EOC is transcriptionally different from that of patients with benign ovarian conditions. This finding may have functional implications because several of the transcripts identified suggest the presence of gene products that may foster cancer cell adherence, growth, and invasion. Several protein products of the transcripts identified have been previously described by others as key components of the EOC microenvironment either within primary tumors or in ascites. Functional validation of our results is beyond the scope of this pilot study, which centered on testing the hypothesis that peritoneal structures may be affected by the presence of EOC before tumor implantation.

It is important to mention that differences noted between the peritoneal structures of individuals with EOC compared to those with a benign condition could be due to the simultaneous expression of various genes by immune cells infiltrating the peritoneum in response to a cancer-driven inflammatory process or by mesothelial cells or fibroblasts naturally part of the

![Fig. 3](https://clincancerres.aacrjournals.org) Selected genes differentially expressed between bST (red bar) and mST (blue bar) with relevance to immune function, angiogenesis, adhesion, or extracellular matrix modulation. Annotations were based on DAVID search program and Genontology discovery tool. The identity of each gene is shown to the side. Ratios are displayed according to the central method for display using a normalization factor (57).
peritoneum or infiltrating myofibroblasts. Obviously, microdissection of individual cells could possibly permit an evaluation of this hypothesis. However, this approach was not feasible in the present study because mRNA amplification at the individual cell level to be utilized for gene expression analysis has not been yet validated in our hands. In silico analysis using electronic Northern and CellSpace suggested that some of the genes identified could be expressed by several immune and nonimmune cells during a chronic inflammatory process underlying some biological redundancy of the cytokine/growth factor network. Indeed, the main thrust of the article was to see whether there were differences in the transcriptional repertoire of unaffected peritoneal tissue. The differences noted between benign and malignant peritoneum were not due to genes expressed by infiltrating EOC cells inasmuch as genes preferentially expressed by EOC, such as CA125, p53, and MOC31, were not significantly increased in the peritoneal specimens of EOC patients. This corroborates the pathologist’s observation that there were no detectable tumor cells in the peritoneal samples. The genes differentially expressed between the peritoneum of individuals with malignant versus benign conditions not only involved inflammatory responses (most likely reflected activation of endogenous or recruited immune cells) but also affected chemotaxis of nonleukocyte stromal cells and tissue remodeling/repair and included integrins and growth factors.

Another important finding was the contrasting homogenous behavior of peritoneal structures in patients with benign pathology compared with the relatively heterogeneous transcriptional profile of patients with EOC particularly in stromal samples (Fig. 1B). This suggests that EOC-associated gene expression profiles may reflect different potentials for influencing their surroundings. This could also be affected by tumor burden of individual patients, which in turn could have important effects on regional and distant progression of this disease. This explorative study was not designed to ask prognostic questions but rather to identify which specimens should be most profitably collected and studied in a large follow-up analysis of EOC patients. It seems that PE and ST samples were most informative compared with blood samples, peritoneal washings, and ascites. In addition, the lack of statistical difference between paired samples suggests that the transcriptional changes observed are the results of a general process and the analysis can be reduced to one sample per patient. Future studies might examine correlations with different disease characteristics. Differences in the genetic profile of PE and ST samples, although not statistically significant because of partial overlapping, may still portray two diverse and complementary biological behaviors: one favoring the adhesion and acute survival of cancer cells (PE), and the other their migration, infiltration, and successful implantation through remodeling of extracellular matrix and expression of growth factors (ST). Therefore, we propose a long-term prospective collection of PE and ST samples in a broad range of patients with EOC to test whether subcategories of EOC could be identified with divergent prognostic significance and, most importantly, different biological behaviors susceptible to distinct molecularly driven therapeutic strategies.

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Peritoneal and Subperitoneal Stroma May Facilitate Regional Spread of Ovarian Cancer

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