Quantitative Analysis of Transforming Growth Factor β1 and 2 in Ovarian Carcinoma


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ABSTRACT
Transforming growth factor β (TGF-β) is an important family of cytokines that may promote tumor growth in vivo through several mechanisms including interference with antitumor T-cell immune responses, alteration of factors in the stroma and matrix, and the promotion of angiogenesis. TGF-β isotypes have been detected in malignant and normal ovarian tissues. We have determined by quantitative immunohistochemistry the density of TGF-β1, TGF-β2, and human leukocyte antigen (HLA) Class I and Class II antigens on malignant cells in paired primary and metastatic specimens from 10 patients with ovarian carcinoma. Cryostat sections of specimens from the carcinomas and from normal ovaries of three women of similar age without ovarian cancer were stained respectively with specific antibodies to TGF-β1, TGF-β2, and HLA Class I and II antigens, and with isotype-matched control antibodies. Antigen density was quantitated blindly as mean absorbance on a SAMBA 4000 image analyzer. TGF-β1 and TGF-β2 were overexpressed in both primary and metastatic tumor specimens in comparison with normal ovarian tissue. No statistical correlation was found between the expression of TGF-β1 or TGF-β2 and HLA class I or HLA class II, which suggests that TGF-β1 isotypes could have effects on the immune system other than down-modulation of these HLA molecules. Furthermore, the lack of association between levels of TGF-β expression and the reduced expression of HLA molecules could suggest that tumor cells expressing both HLA and TGF-β may be suitable targets for adaptive immunotherapy. Additional studies are necessary to determine whether TGF-β expressed by ovarian cancer cells merits evaluation as a therapeutic target.

INTRODUCTION
Ovarian cancer is the fourth most common cause of cancer death in women of the United States. Notwithstanding new technologies, aggressive surgery, and modern chemotherapy, there has been little change in the survival of ovarian cancer patients over the past 15 years (1). The cancer has a typical pattern of spread, with metastasis generally limited to the peritoneal cavity. To improve upon survival, further understanding of the biology of this tumor will be needed. TGF-β is a polypeptide produced by many different cell types, including cells from ovarian carcinoma and normal ovarian tissues (2, 3). TGF-β1 (the proteotypic form) and -β3 isotypes are expressed in adult tissues, whereas TGF-β2 expression occurs mainly during development and differentiation (4). Assembled in an inactive form, the cytokine must be cleaved in the extracellular matrix and on the outer surface of the cell membrane to achieve its activated state (5, 6). TGF-β may interfere with antitumor immune responses by inhibiting the activation of T cells (7) and certain monocytes and by down-regulating major histocompatibility antigens on tumor cells (8). Increased expression of different TGF-β isotypes has been associated with more aggressive tumor behavior and worse prognosis in malignant melanoma (9) and colon cancer (10) and in ovarian cancer (11).

TGF-β isotypes have been detected in ovarian carcinoma specimens using standard qualitative immunohistochemical methods. These studies have suggested that TGF-β is overexpressed in ovarian carcinoma, although quantitative analyses have not been done. The purpose of this study was: (a) to determine by quantitative immunohistochemical analysis the distribution and variability of the staining intensity of TGF-β1 and TGF-β2 isotypes in primary ovarian tumors and their metastases; (b) to determine whether ovarian cancer tissues overexpress TGF-β1 or TGF-β2 in comparison with normal postmenopausal ovarian tissues; and (c) to determine whether the expression of TGF-β1 or TGF-β2 reversely correlates with the expression of HLA class I or II antigens in identical ovarian carcinoma specimens. These studies assume further importance in the context of the development of effective bioimmunotherapeutic strategies for ovarian cancer (12).

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3 The abbreviations used are: TGF-β, transforming growth factor β; HLA, human leukocyte antigen; MOD, mean optical density; DAB, diaminobenzidine; GM-CSF, granulocyte-macrophage colony-stimulating factor; TIL, tumor infiltrating lymphocyte.
MATERIALS AND METHODS

Patients and Specimens. Tissue samples were obtained at the time of surgery for tumor reduction from 10 consecutive patients presenting to the M. D. Anderson Cancer Center for the treatment of advanced ovarian cancer. The patients ranged in age from 55 to 77 years. Patient 1 was Hispanic; the others were Caucasian. None of the patients had a previous or synchronous cancer, and only patient 10 had received prior chemotherapy (paclitaxel, carboplatin, and tamoxifen). All of the other patients underwent laparotomy as initial treatment, and specimens were obtained from the primary tumor on the ovary and from a site of metastasis (the pelvis for patient 1; the omentum for the others). Patients 2, 7, and 10 had stage IV disease, whereas the others had stage IIIC disease. Patient 1 had a mixed mesodermal tumor of the ovary. The others had serous tumors, with areas of transitional cell and clear cell types in patient 6, and areas of transitional cell type in patient 8. Normal ovarian tissue from patients without ovarian cancer (two postmenopausal, one premenopausal) were also obtained for comparison.

Histology. Ovarian tumor specimens free of necrotic tissue were identified from primary and metastatic tumors by a pathologist. Tissues were embedded in polyfreeze tissue freezing medium (Polysciences, Inc., Warrington, PA), snap-frozen in liquid nitrogen, and stored at −70°C. The use of frozen specimens in contrast to paraffin-embedded specimens has the advantage that the unmasking of denatured antigens is frozen specimens in contrast to paraffin-embedded specimens. All of the other patients underwent laparotomy as initial treatment, and specimens were obtained from the primary tumor on the ovary and from a site of metastasis (the pelvis for patient 1; the omentum for the others). Patients 2, 7, and 10 had stage IV disease, whereas the others had stage IIIC disease. Patient 1 had a mixed mesodermal tumor of the ovary. The others had serous tumors, with areas of transitional cell and clear cell types in patient 6, and areas of transitional cell type in patient 8. Normal ovarian tissue from patients without ovarian cancer (two postmenopausal, one premenopausal) were also obtained for comparison.

Antibodies. Affinity-purified rabbit polyclonal IgG antibodies to TGF-β1 and TGF-β2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used as primary antibodies, with rabbit polyclonal IgG (Vector Laboratories, Burlingame, CA) as a control antibody. The following monoclonal antibodies to HLA were used: anti-HLA Class I (W6/32), an IgG2a that recognizes a monomorphic determinant of the HLA Class I molecule (DAKO Corporation, Carpinteria, CA) and anti-HLA Class II (anti-HLA-DR), an IgG1 that recognizes a common framework determinant of the HLA-DR (DAKO).

Immunohistochemistry. Tissue sections were incubated with primary or control antibodies for 2.5 h at room temperature. The optimum concentration of antibodies for staining, as determined by previous experimentation, was 0.1 μg/ml. Tissue sections were then washed and incubated with biotinylated goat antirabbit IgG antibody at a concentration of 1:200 (Vector Laboratories, Inc., Burlingame, CA) for 1 h at room temperature. After extensive washing, avidin-biotin-peroxidase complex (Vector Laboratories) was applied for 30 min. The peroxidase activity was developed with freshly prepared DAB (0.025%), nickel chloride (0.02%), and hydrogen peroxide (0.005%) in PBS. Methyl green (1% in methanol) was used for counterstaining. As controls, adjacent tissue sections from the same block were prepared and stained with isotype-matched irrelevant monoclonal antibodies (IgG1 or IgG2a) at the same concentrations used with the test monoclonal antibodies. To avoid intensity variations, specimens were stained with the test antibody and isotype control in batches. After staining, each specimen was assigned a code number from a random number table, and investigators were blinded to the codes until the completion of the study.

Immunohistochemical Analysis. To evaluate TGF-β and HLA antigen expression, the immunohistochemically stained slides were analyzed using a SAMBA 4000 image analyzer. This system uses an enhanced IBM 486 personal computer with a high-speed digital camera, video monitors, and a calibrated imaging system. The software used was Immunolabeling 4.06 (BioLogics, Inc., Gainesville, VA).

A nuclear-masking technique was enabled by the differing light absorption spectra of the chromogen DAB and the counterstain methyl green. A mask of nuclei, stained with methyl green or DAB, was made under red-filtered light and stored electronically. Masked areas were then analyzed under green-filtered light in which only DAB-stained cells were visible.

Fields for study were identified under a ×20 objective using the “area” mode of analysis. By using a grid system, nine fields containing tumor were chosen from each tumor slide, and nine fields were chosen at random from each slide of normal ovary. Debris, lymphocytes, stromal tissue, and necrotic areas were omitted using the “segmentation” and “remove” options. The color bar value for each specimen was established from the analysis of positive and negative controls. Threshold values and false background color were determined for each specimen individually. Antigen density was quantitated by the computer as MOD and recorded for each specimen. Isotype-matched antibodies assured us that the computer assessed the antigens of interest. Slides of a tumor known to be positive for TGF-β were stained with every batch as positive controls, to confirm the uniformity of staining.

For each patient 12 slides were analyzed: slides of primary tumor from the ovary stained for TGF-β1, TGF-β2, IgG control, HLA I, HLA II, and IgG control; and slides of tumor from the metastatic site stained for the same antigens. Microphotographs were obtained at a magnification of ×250.

Statistical Analysis. MOD values for the test antibodies were corrected for background staining by subtracting the mean MOD value of the appropriate isotype control. The median, 25th and 75th percentiles, and range were calculated for each tumor slide, and pairwise comparisons of means within patients were performed using t tests. Correlations were performed as Pearson product moment correlations. ANOVAs were performed using SAS/STAT procedure MIXED (14), with patient as random effect; logarithmic transformation was done before the ANOVAs were performed to stabilize the variance within groups.

RESULTS

Automated analysis demonstrated that TGF-β was present in all of the tissue specimens examined, including tumor specimens as well as histologically normal specimens from patients without ovarian cancer. Representative photomicro-
graphs of ovarian cancer tissues from patient 9 stained with antibodies reactive with TGF-β1, TGF-β2, and isotype control as shown in Fig. 1. Postmenopausal ovarian tissue showed only minimal staining, and there was no detectable staining with control antibodies. TGF-β1 antigen intensity (expressed as MOD) was greater in primary and metastatic ovarian carcinoma than in normal postmenopausal ovarian tissue: P < 0.012 for primary tumors and P < 0.024 for metastatic tumors (Table 1). The same was true for TGF-β2 antigen intensity, which was significantly higher in primary tumors (P < 0.001) and in metastatic tumors (P < 0.049) than in postmenopausal normal ovarian tissue.

The distribution of staining for TGF-β1 and TGF-β2 in paired primary and metastatic tumor specimens and in normal tissue is shown in Fig. 2. The range of antigen expression within tumor specimens was greater than the range within normal postmenopausal ovarian tissue. There was no statistically significant difference overall in the expression of TGF-β1 or TGF-β2 in primary or metastatic tumor specimens from the same patient, and neither isotype of TGF-β was expressed more than the other (Fig. 3). TGF-β1 and TGF-β2 expression were correlated in both the primary and the metastatic tumor specimens. Although overall differences in expression were not detected, significant correlations were present in certain patients.

The distribution of HLA class I and class II antigen expression in the paired specimens is shown in Fig. 4. Neither HLA class I nor HLA class II expression correlated with the expression of TGF-β1 or TGF-β2 (Table 2).

### Table 1 Corrected MOD values for TGF-β1 and TGF-β2 in primary and metastatic tumor sections and normal ovarian tissue

<table>
<thead>
<tr>
<th>Slide</th>
<th>Fields examined</th>
<th>MOD ± SD (in parentheses)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary tumor</td>
<td>83</td>
<td>2.26 ± 1.06 (0.72–4.96)</td>
<td>&lt;0.012</td>
</tr>
<tr>
<td>Metastasis</td>
<td>85</td>
<td>2.86 ± 1.42 (0.43–6.63)</td>
<td>&lt;0.024</td>
</tr>
<tr>
<td>Normal ovary</td>
<td>18</td>
<td>1.06 ± 0.63 (0.20–2.56)</td>
<td></td>
</tr>
<tr>
<td>TGF-β2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary tumor</td>
<td>86</td>
<td>2.94 ± 1.63 (0.73–7.57)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Metastasis</td>
<td>84</td>
<td>3.55 ± 2.14 (0.16–7.29)</td>
<td>&lt;0.049</td>
</tr>
<tr>
<td>Normal ovary</td>
<td>18</td>
<td>0.84 ± 0.54 (0.09–2.33)</td>
<td></td>
</tr>
</tbody>
</table>

* Tissue was immunohistochemically stained using specific antibodies, DAB, and methyl green and was analyzed under both red and green light to allow electronic masking as described in “Materials and Methods.”

Results represent the mean ± SD (in parentheses) and the range in approximately nine fields from each of 10 ovarian cancer patients and subjects with normal ovaries.

ANOVA after logarithmic transformation, comparing MOD in tumor and in normal ovarian tissue.

### DISCUSSION

TGF-β1 and TGF-β2 mRNA have been documented in ovarian cancer cell lines, and these isotypes in addition to TGF-β3 have been detected in human ovarian carcinoma tissues (11). The protein has been detected in culture supernatants from fresh tumors and established tumor cell lines by ELISA and bioassay (15, 16). Results of qualitative immunohistochemical studies suggest that TGF-β may be overexpressed in ovarian cancer tissue when compared with benign tumors and to normal
ovarian tissue (3). We have used image analysis to quantitate TGF-β1 and TGF-β2 expression, detected by immunostaining. Both antigens were overexpressed quantitatively in ovarian carcinoma when compared with normal postmenopausal ovarian tissue. Staining intensity did not differ in primary and metastatic tumors overall, although differences were seen in some patients.

We detected TGF-β in all of the ovarian tissues examined, whether benign or malignant. Our report differs from another report (17), in which not all of the specimens of ovarian cancer expressed TGF-β. The more frequent detection of TGF-β in our studies could be attributed to our use of: (a) solid specimens from patients who were naïve to chemotherapy in 9 of 10 cases; (b) separate antibodies for the detection of TGF-β1 and TGF-β2; and (c) an automated quantitative method. It is possible that tumor cells present in ascites could differ from solid tumor tissue in the production of TGF-β because of exposure to different cytokines. Automated quantitation using digital image analysis provides a more objective analysis of tissue antigen expression than standard methods of visual scoring or quantitation for comparison either within or between specimens (18–20).

The role of TGF-β in ovarian cancer remains poorly understood. Results of qualitative immunohistochemical studies on normal ovarian tissue sections suggest that TGF-β could be important for cellular differentiation (21–23). Both TGF-β1 and TGF-β2 are expressed in ovarian tissues during all of the phases

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**Fig. 2** Antigen intensity of TGF-β1 and TGF-β2, expressed as corrected MOD, in paired primary and metastatic tumor specimens compared with TGF-β antigen density in normal ovarian tissues. The box plots show the quantitative distribution of TGF-β1 and TGF-β2 isotypes in multiple fields of paired specimens from 10 patients. From top to bottom within each patient column: TGF-β1 (B1) from primary tumor site (P); TGF-β1 from metastatic tumor site (M); TGF-β2 (B2) from primary tumor site; TGF-β2 from metastatic tumor site; the shaded horizontal boxes, the middle 50% of the data (25th to 75th percentile); the white stripes, the median; shaded band areas, normal ovarian tissue MOD values for TGF-β (both TGF-β1 and TGF-β2; 25th to 75th percentile). Raw data were corrected for background staining by subtracting the mean MOD of an adjacent tissue section stained with an isotype control antibody.
of the menstrual cycle but vary in expression at different times of the cycle. Staining for TGF-β1 seems to be pervasive but is most intense in theca and granulosa cell layers in the midluteal phase. In contrast, TGF-β2 seems to be produced only by theca cells in the follicles and small luteal cells (24). There is less variation of TGF-β staining in the postmenopausal ovarian tissues because follicles are senescent. It seems that TGF-β may be involved with the physiological control of the growth of these structures.

The growth-regulating effects of TGF-β in malignant tissues are undergoing intensive evaluation. TGF-β is a bifunctional regulator of cell growth. It stimulates proliferation of mesenchymal cells and inhibits growth of other cell types, primarily epithelial cells (25). The complexity of the issue is emphasized by the contrasting results of in vivo and in vitro experiments. It is possible that dedifferentiation may alter the effect of TGF-β on tumor cell growth, even leading to stimulation of growth in some cases (26). Other factors can also modify the effect of TGF-β in cell growth. It was shown in normal rat intestinal cells transformed with H-ras that this oncogene down-regulates expression of the type II receptor (TGF-βRII), thus rendering the cells resistant to growth inhibition by TGF-β. Although ras overexpression is not a common event in ovarian cancer, loss of the H-ras allele has been reported (27). Also, it has recently been shown that the cytokine GM-CSF can overcome the TGF-β block of cell division by up-regulating expression of cyclin D2 Cdk6 (28). Moreover, GM-CSF is expressed by ovarian cancer cells (29). Additionally, TGF-β may be an angiogenic factor, because TGF-β promotes basement membrane deposition, capillary sprout formation, and recruitment and differentiation of smooth muscle cells (30).

TGF-β has potent immunosuppressive effects: it inhibits T-cell growth and cytokine production, decreases expression of costimulatory ligands, inhibits antigen presentation, and can shift the direction of an immunological response toward Th2 differentiation. TGF-β may promote the survival of immunogenic tumor cells by suppressing the maturation of precytotoxic T cells to cytotoxic effector cells (CD8+ TCRβ+) and the activation of helper T cells (CD4+ TCRβ+; 7, 31, 32).

TGF-β may be responsible, in part, for anergy that is generally exhibited by freshly prepared TILs, unless they are cultured with recombinant interleukin 2 or other appropriate cytokines (33, 34). TGF-β has been shown to have an immunosuppressive effect on TILs from patients with ovarian cancer (7). We have previously demonstrated that HLA class I expression on tumor cells correlates with T-cell infiltration in vivo of ovarian carcinoma and with the ability of these TILs to expand in vitro in response to low concentrations of recombinant interleukin 2 (35). It may be that TGF-β interferes with antitumor immune responses by down-regulating major histocompatibility antigens on tumor cells. Experiments on tumor cell lines have shown that the increase in major histocompatibility antigens induced by IFN-γ may be blocked by prior or simultaneous treatment with TGF-β (8, 36, 37). In contrast, we and others have previously shown that i.p. injection of IFN-γ increased the expression of HLA class I and HLA class II antigens despite the presence of endogenous TGF-β in these patients (12, 38). In the present study, our finding that the intensity of TGF-β1 or TGF-β2 staining did not correlate with the intensity of staining for HLA class I or HLA class II suggests that, in at least some cases, mechanisms other than down-regulation of HLA class I or II may be responsible for the immunosuppressive effect of TGF-β on TILs.
Table 2  Correlation analysis of TGF-β1 or TGF-β2 and HLA I or HLA II in primary and metastatic tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Antigens compared</th>
<th>n</th>
<th>Correlation coefficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>TGF-β1 and HLA I</td>
<td>10</td>
<td>0.314</td>
<td>0.38</td>
</tr>
<tr>
<td>Primary</td>
<td>TGF-β1 and HLA II</td>
<td>9</td>
<td>-0.299</td>
<td>0.44</td>
</tr>
<tr>
<td>Primary</td>
<td>TGF-β2 and HLA I</td>
<td>10</td>
<td>0.073</td>
<td>0.84</td>
</tr>
<tr>
<td>Primary</td>
<td>TGF-β2 and HLA II</td>
<td>9</td>
<td>-0.364</td>
<td>0.34</td>
</tr>
<tr>
<td>Metastatic</td>
<td>TGF-β1 and HLA I</td>
<td>10</td>
<td>0.131</td>
<td>0.72</td>
</tr>
<tr>
<td>Metastatic</td>
<td>TGF-β1 and HLA II</td>
<td>10</td>
<td>-0.464</td>
<td>0.18</td>
</tr>
<tr>
<td>Metastatic</td>
<td>TGF-β2 and HLA I</td>
<td>10</td>
<td>0.064</td>
<td>0.85</td>
</tr>
<tr>
<td>Metastatic</td>
<td>TGF-β2 and HLA II</td>
<td>10</td>
<td>-0.206</td>
<td>0.57</td>
</tr>
</tbody>
</table>

*a* Tissue was stained, and corrected MOD was obtained as in Table 1. Correlation coefficients and P's are the results of Pearson product moment correlations.
The high intensity of staining of TGF-β1 and TGF-β2 in ovarian cancer tissues and the finding that one of these proteins is not expressed significantly more than the other in the paired specimens of these tumors suggests that TGF-β could be used as a target for therapeutic manipulation. Possible therapeutic approaches include monoclonal antibodies directed toward TGF-β (7), antisense oligonucleotides directed against TGF-β (39 – 41), and the use of TGF-β inhibitory compounds such as the aromatic fatty acids, sodium phenylacetate, and phenylbutyric acid (42, 43). Antisense therapy with TGF-β2 specific oligonucleotides in particular have inhibited the growth of intracranial gliomas (39, 40) and mesothelioma (41). Cellular immunosuppressive effects were reversed by the treatment, and the cytotoxic activity of lymph node effector cells in treated animals was increased 3- to 4-fold as compared with controls. In addition, tumor growth has been reversed in human glioma cell lines by treatment with antisense oligodeoxynucleotides of TGF-β2 (39). Intracranial gliomas have been eradicated in vivo by gene therapy with antisense oligodeoxynucleotides to TGF-β2, and the lytic activity of lymph node effector cells from treated animals increased 3- to 4-fold compared with controls (40).

Our data reveal that two TGF-β isotypes are similarly overexpressed. If TGF-β is to be the target of immune or molecular therapeutics, the isotype to be targeted may have to be defined. It is possible that systemic treatments that could inhibit the production or activation of certain isotypes could have detrimental effects. Studies in knockout mice have suggested that TGF-β1 and TGF-β2 have different biological functions. When the TGF-β1 gene is disrupted, animals show no gross developmental abnormalities, but several weeks after birth they succumb to a wasting syndrome characterized by excessive inflammatory response and early death (44, 45). On the other hand, when the TGF-β2 gene is disrupted, mice exhibit a range of developmental defects but lack the wasting syndrome (4). TGF-β2 may be a preferred target until more is known about the effects of targeting the production or the activity of TGF-β1 and TGF-β3 on the adult animal.

In summary, our findings show that TGF-β1 and TGF-β2 are both quantitatively overexpressed on ovarian cancer tissues. The overexpression of TGF-β marks this cytokine as important in ovarian cancer biology, with potential effects on tumor growth, differentiation, angiogenesis, and immune suppression. Additional studies are needed to define the role of TGF-β overexpression in malignant ovarian tumors in vivo.

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