P-Glycoprotein Expression and DNA Topoisomerase I and II Activity in Benign Tumors of the Ovary and in Malignant Tumors of the Ovary, before and after Platinum/Cyclophosphamide Chemotherapy

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ABSTRACT

P-glycoprotein (P-gp) expression and DNA topoisomerase (Topo) II are important variables in multidrug resistant tumor cell lines. The aim of this study was to evaluate P-gp expression and Topo I and II activity in benign and malignant epithelial ovarian tumors. P-gp expression was analyzed immunohistochemically in cryostat sections of fresh tumor specimens. In the same specimens Topo I and II activity were measured by, respectively, relaxation of supercoiled plasmid pBR322 DNA and decatenation of kinetoplast DNA. P-gp expression (range, 5–100% positive staining cells) was found in 3 of 6 cystadenomas, 0 of 2 borderline tumors, 15 of 21 untreated ovarian cancers, and 8 of 13 platinum/cyclophosphamide treated ovarian cancers. Median Topo I and II activity were elevated in malignant tumors compared to benign and borderline tumors. No difference was found between median Topo I activity in untreated ovarian cancer and platinum/cyclophosphamide treated ovarian cancer. High Topo II activity (≥8 × 10² units/mg protein) was more frequent in untreated compared to platinum/cyclophosphamide treated samples. Respectively, 8- and 16-fold differences in Topo I and II activity were found in the malignant tumors. Topo II activity in malignant tumors correlated with Topo I activity (r = 0.36, P < 0.05) and the tumor volume index (r = 0.35, P < 0.05). However, this last weak correlation cannot explain the 16-fold differences in Topo II activity in malignant tumors. Mitotic index and P-gp expression did not correlate with Topo I or II activity. A large variability in P-gp expression and Topo I and II activity was observed in patients with ovarian cancer.

INTRODUCTION

Ovarian cancer is the leading cause of gynecological cancer death in the Western world (1). Chemotherapy is the treatment of choice in these patients when surgical resection is not considered to be curative. Nowadays, first line chemotherapy in ovarian cancer consists of a platinum analogue in combination with Cy³ with response rates of 48 to 76% (2). Other chemotherapeutic agents such as anthracyclines and epipodophyllotoxins have been shown to be active against ovarian cancer with response rates of 23 to 36% (3). However, the majority of patients will die of their disease (4). These clinical data suggest that an intrinsic and acquired resistance to chemotherapy must occur in ovarian cancer. Our aim was to study the expression of P-gp and DNA Topo I and II activity in benign and malignant epithelial ovarian tumors in order to evaluate possible variables related to the resistance of ovarian cancer to drugs such as anthracyclines and epipodophyllotoxins.

In several human tumor cell lines selection for resistance to a single natural product drug resulted in cross-resistance to other natural products, the so-called MDR, which is associated with the increased expression of cell membrane P-gp, resulting in an increased efflux of the natural drugs. Resistance to drugs involved in MDR such as anthracyclines and epipodophyllotoxins can also be associated with alterations in the target of these drugs, Topo II, which mechanism has been called atypical MDR (5, 6). Knowledge of these variables, P-gp and Topo II, in human tumors is increasing but still limited. In recent studies, expression of P-gp in tumor cells measured with immunohistochemistry appeared to be an important adverse prognostic factor concerning response to natural products in different human malignancies (7–9). With respect to ovarian cancer previous studies using Northern and slot blot hybridization techniques showed no P-gp mRNA expression in untreated ovarian cancer (10, 11). Immunological detection of P-gp has been performed in three small series of ovarian carcinoma (12–14) and in one larger series (15). These studies showed conflicting data concerning the expression of P-gp in ovarian cancer. Chan et al. (16) showed in human ovarian cell lines that immunocytochemical detection of P-gp, using a three step immunoperoxidase technique, was as sensitive as Northern blot and more sensitive than standard Western blot. Therefore, we chose to study the expression of P-gp in ovarian tumors using the same immunohistochemical technique.

For the study of atypical MDR Topo II was determined and, because of close relation with Topo II, also Topo I. Topos I and II are nuclear enzymes involved in the regulation of DNA topology and have been identified as the targets of several antitumor agents (17). Topo I for camptothecin and camptothecin derivatives and Topo II for anthracyclines, epipodophyllotoxins, acridines, mitoxantrone, and ellipticine (18–21). Resistance to topoisomerase inhibitors has been attributed to quantitative and/or qualitative changes in both topoisomerases in several cell lines (22–26). Thus far, hardly any data exist on Topo I and II activity in solid human tumors, and no data are available on Topo I and II activity in ovarian cancer.

All the patients from whom tumor samples were taken after chemotherapy received Pt/Cy containing chemotherapy. Until now no data are available to show a relationship between P-gp expression and resistance to platinum or alkylating agents. However, in cell lines conflicting data exist on Topo II in resistance to platinum and alkylating agents. Topo II might have a role in DNA repair by making DNA damage accessible to repair enzymes (27). In cell lines with an acquired resistance to platinum increased and decreased Topo II activity have been described (28, 29). In cell lines with an acquired resistance to alkylating agents increased Topo II activity has also been reported (27, 30). Therefore, we determined Topo I and Topo II activity in residual or relapsed ovarian cancer after Pt/Cy chemotherapy. All these data were correlated with tumor volume and mitotic indices.

Received 4/8/91; accepted 8/19/91.

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1 This study was supported by Grants GUKC 90–18 and GUKC 91–12 from the Dutch Cancer Society.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: Cy, cyclophosphamide; P-gp, P-glycoprotein; Topo, DNA topoisomerase; MDR, multidrug resistance; kDNA, kinetoplast DNA; Pt/Cy, platinum/cyclophosphamide.

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MATERIALS AND METHODS

Monoclonal Antibody, DNA, Cell Lines, and Chemicals. The monoclonal antibody used for P-gp detection was C219 (Centocor Diagnostics, Malvern, PA). Form I kDNA was isolated from the mitochondria of Crithidia fasciculata and purified by CsCl/ethidium bromide centrifugation as described previously (25). The supercoiled dimer of plasmid pBR322 DNA was prepared from Escherichia coli strain HB 101. Plasmid pBR322 DNA was isolated according to the alkaline lysis method and purified by CsCl/ethidium bromide centrifugation as described before (25). Myeloma cell lines with absent (8226S), low (8226Dox 4-6), and high (8226 Dox 40) P-gp expression were kindly provided by Dr. W. Dalton (31). Phenylmethylsulfonyl fluoride was obtained from Merck, Darmstadt, Germany.

Human Material. Tumor specimens were obtained from tumors operated at cooperating hospitals in the northern part of the Netherlands during the period 1987–1990. The tumor collection was supervised by a pathologist. The samples for immunohistochemistry and topoisomerase extraction were immediately frozen in liquid nitrogen and stored at −180°C until further analysis. In two patients untreated ovarian cancer tumor specimens were obtained from two different sites of the same tumor. In one patient untreated ovarian cancer tumor specimens were obtained from the ovarian tumor and a metastasis in the omentum at the same time. In one patient tumor specimens were obtained before and after chemotherapy.

Pathological Characteristics. The tumors were histologically classified according to the WHO classification using paraffin-embedded tissue sections (32). One section per cm tumor diameter was made to obtain a good overall impression of the tumor histology. Tumor volume index and mitotic activity index were measured in the paraffin-embedded sections. The tumor volume index (percentage of malignant epithelial tissue in tumor specimen) was measured by a point counting technique, using a 42-point grid placed on a projection microscope ×200 as described by Baak et al. (33). The mitotic index was calculated by counting the number of mitotic figures in ten high power fields at ×400.

Immunohistochemistry. Cryostat sections were used for immunohistochemistry. Cryostat sections of the tumor specimens were made (6 µm), allowed to dry for 1 h, then fixed in cold acetone (−20°C), and stored at −180°C until examination. For P-gp detection the C219 monoclonal antibody was used as described by Kartner et al. (34). Immunostaining was performed with a three step immunoperoxidase technique using rabbit anti-mouse antibody and swine anti-rabbit peroxidase conjugated antibody. The peroxidase staining was performed by two pathologists (H. H. and A. G.). The percentage of positive staining was determined, an overall percentage of positive cells was expressed in units. One unit Topo I activity was defined as the lowest concentration of protein capable of relaxation of 0.9 µg supercoiled plasmid pBR322 DNA. One unit Topo II activity was defined as the lowest concentration of protein capable of complete decatenation of 0.2 µg kDNA. Levels of Topo I and II activity in tumor extracts were compared by serially diluting extracts with the same protein concentration. All experiments were performed in duplicate. To avoid contamination by connective tissue, the epithelium of the cystadenomas and borderline tumors were dissected away from the cyst wall and used for further analysis. To correct for possibly different contamination of the tumor samples with RBC, which show an undetectable Topo I and II activity, the concentration of hemoglobin in all the tumor extracts was measured by a second derivative spectrophotometric assay for hemoglobin in serum (36). The percentage of protein extracted from RBC in the tumor could be calculated after dividing the hemoglobin concentration in the tumor extracts by the hemoglobin concentration found in a whole blood extract.

Statistical Analysis of the Difference in the Topo I Activity. The percentage of P-glycoprotein expression was detected in a whole blood extract.

RESULTS

Tumor Pathology, Mitotic Index, and Tumor Volume Index. Six patients had benign cystadenomas, 2 patients had borderline disease, 21 patients untreated ovarian cystadenocarcinoma, 6 patients had residual disease at second look operation after chemotherapy, and 7 patients had recurrent disease after pathologically confirmed complete remission. For specification of chemotherapeutic regimens in these patients, see Table 1. Mean mitotic index and mean tumor volume index are shown in Table 2.

Table 1. Chemotherapy used in patients with residual or recurrent disease

<table>
<thead>
<tr>
<th>Patients</th>
<th>Chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 res.d.</td>
<td>CC (6×)</td>
</tr>
<tr>
<td>2 res.d.</td>
<td>CC (6×)</td>
</tr>
<tr>
<td>3 res.d.</td>
<td>CC (3×)</td>
</tr>
<tr>
<td>4 res.d.</td>
<td>CC (6×)</td>
</tr>
<tr>
<td>5 res.d.</td>
<td>CC (6×)</td>
</tr>
<tr>
<td>6 res.d.</td>
<td>CC (6×)</td>
</tr>
<tr>
<td>7 res.d.</td>
<td>CHAP (6×), Cy (5×)</td>
</tr>
<tr>
<td>8 res.d.</td>
<td>CC (6×)</td>
</tr>
<tr>
<td>9 res.d.</td>
<td>CP (5×), CC (2×)</td>
</tr>
<tr>
<td>10 res.d.</td>
<td>CC (9×)</td>
</tr>
<tr>
<td>11 res.d.</td>
<td>CC (6×)</td>
</tr>
<tr>
<td>12 res.d.</td>
<td>CC (6×)</td>
</tr>
<tr>
<td>13 res.d.</td>
<td>CC (6×), P/Vp (3×)</td>
</tr>
</tbody>
</table>

* Res.d., residual disease; rec.d., recurrent disease; CP, cyclophosphamide, cisplatin; CC, cyclophosphamide, carboplatin; CHAP, cyclophosphamide, hexamethylmelamine, Adriamycin, cisplatin; P/Vp, cisplatin i.v., etoposide i.p.; n×, number of cycles.
cells, median percentage of C219 positive staining tumor cells (range). Volume index (percentage of malignant epithelial tissue) ± SD; % C219 positive untreated cancer; Res./rec., residual and recurrent disease; TVI, mean tumor volume index (percentage of malignant epithelial tissue) ± SD; % C219 positive cells, median percentage of C219 positive staining tumor cells (range).

Table 2 Mitotic index, tumor volume index, and P-glycoprotein expression in ovarian tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>MI*</th>
<th>TVI</th>
<th>% of C219 positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign (n = 8)</td>
<td>23.8 ± 19.7</td>
<td>48.4 ± 19.9</td>
<td>7.5 (0-50)</td>
</tr>
<tr>
<td>Untr.ca. (n = 22)</td>
<td>16.8 ± 16.3</td>
<td>50.1 ± 24.5</td>
<td>5 (0-30)</td>
</tr>
</tbody>
</table>

* MI, mean mitotic index (mitotic figures/10 high power fields) ± SD; Untr.ca., untreated cancer; Res./rec., residual and recurrent disease; TVI, mean tumor volume index (percentage of malignant epithelial tissue) ± SD; % C219 positive cells, median percentage of C219 positive staining tumor cells (range).

Table 3 Intensity and location of C219 signal in C219 positive tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>C219 intensity</th>
<th>C219 location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign (n = 3)</td>
<td>++</td>
<td>c/m</td>
</tr>
<tr>
<td>Untr.ca. (n = 16)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Res./rec. (n = 8)</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

+ = cytoplasmic; c/m, cytoplasmic and membrane bound; m, membrane bound; Untr.ca., untreated cancer; Res./rec., residual and recurrent disease; for specification of C219 intensity, see "Materials and Methods."

Immunohistochemistry. The results of C219 immunohistochemistry are shown in Tables 2 and 3, and Fig. 1. The interobserver variability between the two pathologists was always <5%. In 16 of the 21 untreated ovarian carcinomas variable P-gp expression (≥5% C219 positive tumor cells) was found with a median percentage of positive staining cells in the 16 positive tumors of 10 (range, 5–50). In the specimens of one patient 30% of the tumor cells in the ovary were positive, while 5% of the tumor cells in the omental metastasis were positive. In one patient 5% of the tumor cells stained positive before and after chemotherapy (platinum, Cy). In 8 of 13 chemotherapeutically treated ovarian carcinomas P-gp expression was found (median percentage of positive staining cells, 12.5; range, 5–30). Two patients with recurrent disease, who also received MDR related chemotherapy, showed P-gp expression (one patient 10% + cells, cytoplasmic; one patient 20% ++ cells, cytoplasmic and membrane bound). For P-gp expression, neither the percentage of positive cells nor the intensity of staining correlated with histological type, Topo I activity, or Topo II activity. In the large majority of the C219 positive malignant tumors (19 of 24) the intensity of the C219 signal was comparable to the intensity of staining of the 8226DOX4-6 cell line (+). In 19 of the 24 C219 positive malignant tumors the staining pattern was cytoplasmic.

Topo I and II Activity. Storage of the tumor specimens at −180°C for 6–9 months did not influence Topo I or II activity. Tumor extracts could not be stored at −20°C because then a significant decrease in Topo II activity occurred within 7–8 days. All Topo I and II assays of the same tumor extracts were performed in duplicate and were highly reproducible. In two patients specimens from two different sites of the same tumor showed the same Topo I and II activity. In one patient biopsies were taken from the tumor of the ovary and metastatic tumor in the omentum at the same time. Topo I activity was 4-fold higher in the metastatic tumor, while Topo II activity was 2-fold lower in the metastatic tumor. From other patients only one tumor specimen was available for Topo I and II determination. The median and range of Topo I and II activity of the different groups are shown in Table 4. For individual Topo I and II activities, see Figs. 2 and 3. Topo I activity in treated plus untreated ovarian cancer (median, 4.0 × 10⁴ units/mg protein; range, 2.0–16.0) was higher (P < 0.01) compared to the Topo I activity in the group of benign cystadenoma plus borderline disease (median, 0.5 × 10⁴ units/mg protein; range, 0.4–1.6). No significant differences were found in Topo I activity in untreated ovarian cancer compared to Topo I activity in residual plus recurrent ovarian cancer. An 8-fold range was found for highest and lowest Topo I activity in all malignant tumors (2–16 × 10⁴ units/mg protein). Topo II activity was elevated in the group of treated plus untreated ovarian cancer (median, 4.0 × 10³ units/mg protein; range, 1.0–16) compared to the group of benign cystadenoma plus borderline disease (detectable, but too low to quantitate). The incidence of a high (≥8 × 10² units/mg protein) Topo II activity was higher in untreated ovarian cancer compared to residual plus recurrent ovarian cancer (P < 0.05). A 16-fold range was found for highest and lowest Topo II activity in malignant tumors (1–16 × 10² units/mg protein). In one patient tumor specimens, taken before and after chemotherapy (cisplatin, cyclophosphamide), showed equal Topo I and II activity. Weak rank correlations were found for Topo II activity in all malignant tumors with the tumor volume index (r = 0.35, P < 0.05) and for Topo II activity with Topo I activity (r = 0.36, P < 0.05) (Fig. 4). No rank correlation was found for Topo I or II activity with the mitotic index. In the tumor extracts the percentage of protein from RBC in the tumor ranged from to 0 to 5%. Considering these very low percentages in all the tumor extracts no correction for contamination of the tumor extracts with protein from RBC was performed.

DISCUSSION

Our results show a widely differing expression of P-gp in benign as well as in malignant ovarian tumors. Malignant tumors arising in organs naturally expressing high levels of P-gp are often intrinsically resistant to MDR related chemotherapy. Previously, no P-gp expression was found in normal human

![Fig. 1. P-gp expression (percentage of C219 positive staining tumor cells) in ovarian tumors. aden, cystadenoma; bord, borderline disease; un.ca, untreated cancer; res.d, residual disease; rec.d, recurrent disease.](image_url)

Table 4 Median and range of Topo I and II activity in ovarian tumors

<table>
<thead>
<tr>
<th>Tumors</th>
<th>No.</th>
<th>Topo I</th>
<th>Topo II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>8</td>
<td>0.5 (0.4–1.6)</td>
<td>a²</td>
</tr>
<tr>
<td>Untr.ca.</td>
<td>22</td>
<td>4.0 (2.0–16.0)</td>
<td>7.5 (1.0–16.0)</td>
</tr>
<tr>
<td>Res./rec.</td>
<td>13</td>
<td>4.0 (2.0–16.0)</td>
<td>2.0 (1.0–8.0)</td>
</tr>
</tbody>
</table>

* × 10⁴ units/mg protein.
* × 10³ units/mg protein.
* a, detectable, but too low to quantitate; Untr.ca., untreated cancer; Res./rec., residual and recurrent disease.
cystadenoma; bord, borderline disease; un.ca, untreated cancer; res.d, residual disease; rec.d, recurrent disease.

... of electro-...sies of tumor tissue required for the various types of electro-...of P-gp was as sensitive as Northern blot and more sensitive...ovarian tumors and in 16 of 21 untreated ovaries (37, 38). In our study P-gp expression was found in 3...disease; rec.d, recurrent disease.

In our study P-gp expression was found in 3 of 6 benign epithelial ovarian tumors and in 16 of 21 untreated malignant tumors. Our results with an indirect immunohistochemical technique are in contrast with previous studies, using Northern and slot blot hybridization techniques, in which no P-gp mRNA expression was found in untreated ovarian cancer (10, 11). Chan et al. (16) showed in human ovarian cell lines with the MDR phenotype that immunocytochemical detection of P-gp was as sensitive as Northern blot and more sensitive than standard Western blot. Perhaps homogenization of biopsies of tumor tissue required for the various types of electrophoretic analyses may produce false negative results in tumor biopsies despite the presence of small subpopulations of P-gp bearing cells. Our results are also in contrast with the results of Rubin et al., who found P-gp expression in only 4 of 57 patients with ovarian cancer. However, Rubin et al. used a two step immunoperoxidase technique, which provides less signal amplification than the three step immunoperoxidase technique used in this study. Recently, Noonan et al. (39) also found a relatively high incidence of P-gp expression in 21 of 26 untreated malignant ovarian tumors with a sensitive detecting method, based on the polymerase chain reaction. Variations in detecting P-gp expression by different techniques were also found in human breast cancer (40, 41). Our data suggest that, in contrast to earlier conclusions, previous treatment with MDR related drugs is not necessary for P-gp expression in ovarian cancer (12). The intensity of the C219 staining in the majority of the malignant tumors in our study was low and located in the cytoplasm. In cell lines, lower degrees of drug resistance to natural products were associated with staining at cytoplasmic sites, while higher degrees of resistance appeared to be associated with membrane bound staining (42). However, studies in soft tissue sarcomas and acute leukemias showed that any immunohistochemically detectable P-gp expression was clinically relevant (7, 8).

Over the last years much knowledge is gathered on topoiso...erases and drug resistance in cell lines, but little information exists on these nuclear enzymes in human neoplasms, especially in solid malignant tumors. Recently, Holden et al. (43) measured Topo I and II activity in six human neoplasms and nine normal tissue samples. In contrast to our findings, Holden et al. found equal Topo I and II levels in neoplastic specimens, such as lymphomas and breast and thyroid carcinoma, and in normal nonproliferating tissues, such as spleen and small intestine. Nelson et al. (44) found in rat prostatic adenocarcinomas higher Topo I and II activity in malignant parts of the prostate than in benign parts. This is in agreement with the present study in which higher Topo I and II activities in malignant ovarian tumors were found compared to benign tumors. Hsiang et al. (45) found with immunoblotting that Topo II activity in untreated colorectal tumors varied from undetectable to elevated, while Topo II activity was low in normal colorectal mucosa. In cultured cell lines Topo II activity is cell cycle dependent, and in resting cells there is a lower level of Topo II activity (46). In this study no correlation was found between mitotic index with Topo II levels in the malignant ovarian tumors. This may be due to a high number of resting cells in solid tumors such as ovarian cancers, but the mitotic index is also a relatively rough reflection of the proliferative status of a malignant tumor. However, high Topo II activity may also be an independent parameter in ovarian carcinoma. A weak correlation was found between tumor volume index and Topo II activity, while the tumor volume index did not differ between untreated and Pt/Cy treated malignant ovarian tumors. This correlation was too weak to explain the 8- and 16-fold differences in Topo I and II activity between the individual malignant tumors. Potmesil et al. (46) suggested that the low levels of Topo II found in chronic lymphocytic leukemia cells offered an explanation for the ineffectiveness of doxorubicin treatment in patients with chronic lymphocytic leukemia. The differences found in Topo II activity between the malignant ovarian tumors in combination with the heterogeneity in P-gp expression may offer an explanation for the varying responses to MDR drugs in ovarian cancer patients (2-4). The increased incidence of low Topo II activity found in this study in Pt/Cy treated tumors perhaps explains lower response rates to Topo II targeted drugs after first line chemotherapy (4).

In cell lines conflicting data exist on the role of Topo II in resistance to platinum and alkylating agents (28-30). Most Pt/
Cy treated malignant ovarian tumors in this study showed a low Topo II activity. Lower Topo II activity in Pt/Cy treated tumors can perhaps be explained by selection of tumor cells with lower Topo II activity by Pt/Cy treatment or may be reflecting a lower metabolic activity or a decreased fraction of tumor cells in S phase in these tumors after Pt/Cy treatment.

The increased Topo I activity in malignant ovarian tumors makes Topo I an interesting target for chemotherapy in ovarian cancer. Giovannella et al. (47) showed that Topo I measured with immunoblotting was elevated in advanced stages of human colon adenocarcinoma and in xenografts of colon cancer carried by immunodeficient mice, compared to normal mucosa. A synthetic analogue of camptothecin was found to be highly effective against these xenografted human colon cancers with high Topo I activity levels.

In conclusion, our observations confirm a possible role for P-gp expression in intrinsic drug resistance to natural drugs in ovarian cancer. Topo I and II activity were higher in malignant ovarian tumors compared to benign and borderline tumors. Topo I and II activity were higher in all malignant ovarian tumors, drugs targeted at the Topo I enzyme could be interesting for further studies. Determination of P-gp expression, as well as Topo I and Topo II activity might help in the future to select the proper treatment for the individual patient.

ACKNOWLEDGMENTS

We acknowledge all participating gynecologists and pathologists for their help in collecting tumor samples.

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