Expression of a Human Multidrug Resistance Gene in Ovarian Carcinomas

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ABSTRACT

Expression of the human MDR1 gene has been shown to confer the multidrug resistance (MDR) phenotype to sensitive cells. To investigate the possible contribution of the MDR phenotype to chemoresistance in ovarian carcinoma, we have analyzed MDR1 gene expression in fresh carcinoma specimens from 50 patients. Fifteen received chemotherapy before surgery and were judged as poor responders. Thirty-five patients did not receive any drug before surgery. Control tissues were lymphocytes from 7 patients. Total RNAs were analyzed by Northern and slot blot hybridization techniques using human MDR1 complementary DNA and human \( \gamma \)-actin complementary DNA probes sequentially as qualitative and quantitative controls. MDR1 transcripts (45 kilobases) were observed in the RNA preparations obtained from 3 of 10 patients who were treated with doxorubicin or vincristine, 2 drugs known to select the MDR phenotype \textit{in vitro}. In 40 other RNA preparations obtained from 35 untreated patients and 5 patients treated exclusively with cyclophosphamide and cis-platinum, no transcript could be detected. Using the exact Fisher test, the difference between the 2 groups was found to be significant \((P < 0.01)\). The three tumors with elevated MDR1 expression did not show MDR1 DNA amplification. Our study suggests that, in spite of the weak occurrence of the MDR process in patients with ovarian cancers, MDR1 expression can be related to previous treatment with doxorubicin or vincristine. These results favor the expression of the MDR1 gene as one of the determinants involved in the acquired chemoresistance of ovarian cancers.

INTRODUCTION

Several mechanisms can be involved in the development of drug resistance. MDR\(^1\) has been studied in cancer cells grown in tissue culture and selected to resistance to therapeutic agents (for reviews, see Refs. 1 and 2). Different groups have demonstrated that the genome of \textit{in vitro}-selected MDR cells contain DNA sequences coding for proteins involved in chemoresistance to unrelated drugs (anthracyclines, \textit{Vinca} alkaloids, actinomycin D). Several DNA sequences are amplified and expressed in MDR cells (3, 4), including the human MDR1 gene which plays a major role in the acquisition and maintenance of the MDR phenotype (3). This gene was cloned, sequenced, and expressed in tissue culture and selected to resistance to therapeutic agents (for reviews, see Refs. 1 and 2). Different groups have demonstrated that the genome of \textit{in vitro}-selected MDR cells contain DNA sequences coding for proteins involved in chemoresistance to unrelated drugs (anthracyclines, \textit{Vinca} alkaloids, actinomycin D). Several DNA sequences are amplified and expressed in MDR cells (3, 4), including the human MDR1 gene which plays a major role in the acquisition and maintenance of the MDR phenotype (3). This gene was cloned, sequenced, and shown to encode a \( M, 170,000 \) membrane glycoprotein, known as P-glycoprotein or gp170 (5). MDR1 cDNA probes as well as monoclonal antibodies directed towards gp170 constitute useful tools for the detection of MDR expression.

The involvement of the MDR1 gene in clinical drug resistance is currently under investigation. Fojo \textit{et al.} (6) have shown that the MDR1 gene is overexpressed in normal tissues such as adrenal, kidney, colon, and in some cancers derived from them. Furthermore these cancers are known to be refractory to cytotoxic drugs thus suggesting an important role of MDR1 gene in intrinsic chemoresistance (7). An increase in MDR1 transcript levels or in gp170 production has also been shown in sarcoma (8) and neuroblastoma (6). Recently a study carried out on 400 fresh tumors indicates that the MDR1 gene is overexpressed in several types of tumors and may contribute to both intrinsic and acquired resistance in human cancers (9).

With respect to ovarian cancers, previous studies have been performed on only small series of ovarian cancers. Bell \textit{et al.} (10) found that 2 of 5 treated tumors exhibited an overproduction of gp170 while in another series of 6 tumors, no MDR1 overexpression could be detected (11). Combinations including cis-platinum, doxorubicin, alkylating agents such as cyclophosphamide, and \textit{Vinca} alkaloids have been shown to be effective in the treatment of ovarian cancers (Ref. 12; for a review see Ref. 13). The cis-platinum combination regimens produce an overall response rate of 60–70\% in patients with an advanced stage of the disease (Federation Internationale de Gynecologie et d’Obstétrique stages III and IV) (14). Nevertheless the complete response rate as judged by pathological examination is only 20–30\%, and the 5-year survival remains below 10\%. The patients who relapse will not be cured whatever the salvage therapy used (14). These clinical data suggest that ovarian cancers show an acquired chemoresistance. Depending on the drug treatment adopted, possibilities for clinical drug resistance may include: a MDR1 overexpression; an increase in intracellular levels of glutathione (15); an increase of metallothionein; variations of DNA repair and of topoisomerases activities (16); a non-MDR1-mediated drug accumulation (2).

In this study we analyzed MDR1 gene expression in 50 surgical specimens obtained from patients with ovarian cancers. Fifteen of them received primary chemotherapy while 35 received no chemotherapy. The data suggest a role for expression of the MDR1 gene in the acquisition of resistance to doxorubicin in ovarian cancers.

MATERIALS AND METHODS

Carcinoma and Control Specimens. Carcinoma specimens were obtained by surgical excision from 34 ovarian carcinomas diagnosed at the Institut Gustave Roussy and 16 specimens analyzed at the National Cancer Institute obtained from the Mayo Clinic, the University of Alabama, and the University of Vienna during the period 1982–1987. Normal cell samples were lymphocytes obtained from 7 of the above patients and were fractionated in a Ficoll-Hypaque gradient. All the tissue specimens and cell samples were immediately frozen and stored in liquid nitrogen while the malignancy of the tumor was assessed according to standard institutional procedures. Detailed clinical data were available for most of the patients. Thirty-five patients did not receive any therapy other than surgery when the specimen was removed, while 15 patients had received chemotherapy before surgical excision. All of these 15 patients exhibited macroscopic residual tumor larger than 2 cm and were judged as poor responders to chemotherapy. In these cases, the drug regimen was based on cis-platinum-cyclophosphamide for 5 patients, on cis-platinum-cyclophosphamide with doxorubicin for 10 patients (plus vincristine for 1 patient). In 2 cases, it was possible to compare tumor specimens taken before and after chemotherapy (cis-platinum-cyclophosphamide).

Cell Lines. KB-3-1, a drug-sensitive parental KB cell line and a colchicine-resistant subline, KB-8-5, have been described elsewhere (17). These lines were used as standard material for quantification of...
**MDR1 transcript levels as described recently (9).**

A line IGR-OV1, OV1/p, has been established (18) from an untreated stage III ovarian carcinoma of the series. From this line a vincristine-resistant line, OV1/vincristine, was selected in vitro; it showed a relative index of resistance of 800 and an overexpression of the MDR1 gene of about 25-50-fold with no DNA amplification (19), as compared to OV1/p.

Preparation of DNA and RNA. DNA and total RNA were prepared from tumor specimens, cell lines, and lymphocytes kept frozen at -70°C, according to the classical method of Maniatis et al. (20).

**Measurement of MDR1 Gene Transcript Levels.** Ten μg of undegraded total RNA per sample were fractionated in a 1.2% formaldehyde-agarose gel and transferred to Gene Screen Plus membranes. In slot blot experiments RNA was denatured in formaldehyde and spotted onto nitrocellulose in 4-fold dilutions (10, 3, 1, and 0.3 μg). The membranes were hybridized with 10 ng/ml 32P-MDR1-labeled probes (specific activity, 2-4 x 10^6 cpm/μg DNA) in 50% formamide-5 x SSC-1% SDS-5 x Denhardt’s-20 mm sodium phosphate, pH 6.8, containing 10 μg/ml salmon sperm DNA. The human MDR1 probes used were cDNA probe HDR5A encompassing coding regions of the gene (21) and probe HDR10 covering 5’ coding and noncoding sequences (22). After hybridization, blots were washed in 0.75 x SSC-1% SDS at 65°C for a total of 1 h followed by two 20-min washes with 0.1 x SSC-0.1% SDS at room temperature. Blots were rehybridized with a human actin probe which provided a qualitative and quantitative control of the RNA preparations. Blots were exposed for various periods of time to XAR5 film. The levels of MDR1 expression were determined by densitometry of the autoradiograms (Chromoscan 3 Joyce Loebl).

First, to test the quality of each RNA tumor sample, total RNAs were compared on Northern blots to OV1/vincristine RNA which exhibits a high MDR1 RNA signal. Second, to quantitate levels of MDR1 transcripts, RNAs from the ovarian samples were compared with RNA from the KB-8-5 subline, which is 3-6-fold resistant to doxorubicin and vincristine and the drug-sensitive KB-3-1 cell line in a slot blot assay. The signal intensity of 10 μg of KB-8-5 total RNA has been assigned an arbitrary value of 30 units (9). Specimens were classified in 3 groups: "undetectable" (0-1 unit); "low positive" (2-29 units); and "positive" (≥30 units) (9).

**MDR1 DNA Content of Tumors.** The tumors which exhibited a positive signal for MDR1 gene expression were also analyzed for amplification of the MDR1 gene. Southern transfer analysis was performed as follows: 10 μg of genomic DNA were restricted with EcoRI, fractionated on a 1% agarose gel, and transferred to Gene Screen Plus membranes. Hybridization was performed with the MDR1 cDNA probes and the β-globin pseudogene was a quantitative control.

**RESULTS**

Total RNA from 50 ovarian carcinoma and 7 lymphocyte specimens, showing no evidence of degradation in gels stained by ethidium bromide, were analyzed by Northern and slot blot hybridizations. Northern blot analysis of the OV1/vincristine line (19) revealed a 4.5-kilobase band corresponding to the MDR1 gene transcript (Fig. 1). In order to rule out cross-hybridizations with the related MDR2 gene (4), which has not been shown to confer drug resistance, the Northern blots were hybridized with both the HDR5A insert which encompasses a large portion of the coding region and the HDR10 insert which covers the 5’ region of the human MDR1 cDNA. The 4.5-kilobase signals obtained on Northern blots were of similar intensity, consistent with the conclusion that this signal corresponded primarily to RNA transcribed from the MDR1 gene. Table 1 presents the clinical characteristics and treatment of the patients whose tumors had been analyzed.

No expression of the MDR1 transcript could be detected either in the 35 untreated patients or in 6 lymphocytes specimens from these patients. Among the 15 treated tumors, 2 exhibited positive and 1 showed low positive (20 units) MDR1 transcript levels as measured using slot blot analysis. The three tumors with elevated MDR1 expression did not show MDR1 gene amplification (data not shown). Figs. 1 and 2 present representative Northern and slot blots. A lymphocyte specimen from a patient exhibiting a positive tumor gave an undetectable MDR1 RNA signal. It is noteworthy that the three tumors with elevated MDR1 expression had all been treated by a regimen that included doxorubicin. One of them had been also treated with vincristine. Table 2 summarizes the clinical status and treatment of the corresponding patients. No relationship between the administered doxorubicin dose and the level of MDR1 gene transcript was found. The 3 of 10 tumors treated by

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**Table 1 Clinical characteristics of the patient group**

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Mean age (yr)</th>
</tr>
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<tbody>
<tr>
<td>50</td>
<td>52 (25-76)</td>
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**Stage (FIGO)**

<table>
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<tr>
<td>I-II</td>
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</tr>
<tr>
<td>III</td>
<td>26</td>
</tr>
<tr>
<td>IV</td>
<td>4</td>
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**Histology of tumors**

<table>
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</thead>
<tbody>
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</tr>
<tr>
<td>Mucinous/endometroid</td>
<td>12</td>
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<tr>
<td>Other</td>
<td>6</td>
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</table>

**Chemotherapy before surgery**

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>No</td>
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<td></td>
<td>35</td>
</tr>
<tr>
<td>Yes</td>
<td>15</td>
<td></td>
<td>15</td>
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</tbody>
</table>

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* Stage and age available for 34 patients only. FIGO, Federation Internationale de Gynécologie et d'Obstétrique.
* Histology of tumors available for 39 patients only.
* For two patients treated by cyclophosphamide-cis-platinum, a tumor specimen taken before and after chemotherapy was available.
doxorubicin or vincristine were compared to the 0 of 40 tumors either untreated or cyclophosphamide-cis-platinum treated. Using an exact Fisher test this difference was found to be significant (P < 0.01).

In addition, for 2 of the 5 cyclophosphamide-cis-platinum-treated patients, specimens were obtained before and after chemotherapy: all MDR1 transcript signals were undetectable and no variation, even slight, was observed.

DISCUSSION

The experiments described herein address the question of the detection of MDR1 gene expression in human ovarian cancers and the contribution of this gene to the process of drug resistance in patients' tumors. Among 50 specimens obtained from both untreated and treated patients, only 3 exhibited significant MDR1 mRNA levels, 2 as positive and 1 as low positive (9). It is noteworthy that the transcript levels of the MDR1 gene are low as compared with those obtained in MDR human ovarian cell lines selected in vitro (19). Our data address the crucial question of whether low levels of MDR1 gene expression might be responsible for clinical chemoresistance through a drug efflux-pump mechanism. On theoretical grounds, the expression of a drug-resistance mechanism at low levels, so that cells are only a few-fold resistant to drugs, should make tumors resistant to chemotherapy. The association of MDR1 gene expression with prior treatment history in ovarian cancer suggests that this expression was selected for by the clinical exposure to doxorubicin and/or vincristine.

Our data show that MDR1 overexpression does not occur in untreated ovarian cancers. This is compatible with the data of Fredericks et al. (11) undertaken on a smaller series. It is important to note that the number of specimens that are MDR1 positive in our series is significantly higher (P < 0.01) in the group of patients treated with a regimen that includes either doxorubicin or vincristine. These known agents are selected to select multidrug resistant cells in vitro. Moreover, our data are in agreement with those of Bell et al. carried out on a series of 5 patients who had undergone chemotherapy before surgery (10). The authors showed that the 2 tumors exhibiting overproduction of gp170, as revealed in Western blots using monoclonal antibodies directed against gp170, had been treated with doxorubicin. In addition one of the positive tumors did not express gp170 initially after a treatment with melphalan but became gp170 positive following 3 courses with doxorubicin. From our data it appears that MDR1 expression can be obtained with low cumulative doses of doxorubicin, far lower than the cardiotoxic level of this agent (Table 2). Verapamil, a calcium channel blocker, has been shown to overcome the MDR phenotype (23). This property has led Ozols et al. (24) to use this compound with doxorubicin in 8 refractory patients with ovarian cancers; no objective response was obtained in this study. The rare occurrence of MDR1 expression in these cancers as well as the fact that verapamil serum levels were probably too low to have any inhibitory effect on the multidrug transporter might account for this apparent lack of modulation of chemoresistance in patients.

Of 10 doxorubicin-treated patients, 7 did not exhibit MDR1 gene expression. Other mechanisms such as the activities of topoisomerase II (16) or glutathione-metabolizing enzymes (25) might be involved in the chemoresistance exhibited by these particular tumors as well as in the 5 cyclophosphamide-cis-platinum treated tumors.

In conclusion, our study suggests that in spite of the low or infrequent occurrence of MDR1 gene overexpression in patients with ovarian cancers, this molecular determinant can be related to a treatment that includes doxorubicin or vincristine. These results favor a role for MDR1 gene as one of the molecular determinants implicated in the acquired chemoresistance of ovarian cancers. They suggest that the development of nontoxic inhibitors of the multidrug transporter encoded by the MDR1 gene might be useful in the treatment of a particular subgroup of ovarian cancers which have an activation of the MDR1 gene.

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