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Decreased levels of topoisomerase IIα in human renal cell carcinoma lines resistant to etoposide

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Abstract Renal cell carcinoma (RCC) displays strong resistance against many chemotherapeutic drugs. Overexpression of P-glycoprotein (Pgp) appears to be part of this resistance. The involvement of another resistance mechanism, involving the decreased activity of DNA topoisomerase II (topoII), remains uncertain. By culturing the human RCC lines RC2 and RC21 in the presence of increasing concentrations of etoposide, we derived the variant sublines RC2E, RC21A and RC21E, that had acquired approximately 30-, 60- and 90-fold resistance to this drug respectively. RC2E, RC21A and RC21E were approximately 50-, 5- and 400-fold cross-resistant to doxorubicin respectively. RC2E and RC21E also showed cross-resistance (approximately 200- and 3500-fold respectively) to vinblastine. Quantitative differences in *MDR1* and Pgp expression (elevated in RC2E and RC21E) and topoIIα (reduced in RC21E and RC21A) were demonstrated using Western blotting and the reverse transcriptase/polymerase chain reaction. Decreased amounts of topoIIα were reflected in a reduced activity of RC21A and RC21E as measured by unknotting phage P4 DNA. Qualitative changes of the topoIIα gene, such as point mutations in the motif B/DNBS and DNA-binding regions, or differences in methylation status of the promoter gene of RC21E, were not found. These cell lines represent a model of a solid tumor in which overexpression of Pgp, a combination of increased Pgp and decreased topoIIα, and a decrease of topoIIα are represented.

Key words DNA topoisomerase II · Antineoplastic agents · Drug resistance · Cultured tumor cells · Renal cell carcinoma

Abbreviations *RCC* renal cell carcinoma · *MDR* multidrug resistance · *Pgp* P-glycoprotein · *topo* topoisomerase · *GAPDH* glyceraldehyde-3-phosphate dehydrogenase · *SSCP* single-stranded conformational polymorphism · *RT-PCR* reverse transcriptase/polymerase chain reaction · *MTT* microtiter tetrazolium test · *DTT* dithiothreitol · *at-MDR* altered-topoisomerase MDR

Introduction

Nearly 30% of patients with renal cell carcinoma (RCC) present with metastases at the time of diagnosis. As surgical treatment will be effective only for localized disease, curative therapy is not possible for these patients. Presently available chemotherapeutic drugs, administered according to standard scheduling, fail to achieve response rates of more than 7%, which might even partly represent an indirect cytotoxic effect (Yagoda et al. 1995). The relative insensitivity of tumor cells to a broad range of cytotoxic agents is called multidrug resistance (MDR). Insights into the mechanisms responsible for MDR should contribute to the development of more effective chemotherapeutic treatments for RCC. One of the best known resistance mechanisms implicated in MDR of RCC consists of overexpression of the *MDR1* gene and its product P-glycoprotein (Pgp), an energy-dependent multidrug transmembrane transporter that prevents intracellular accumulation of cytotoxic drugs by rapidly extruding them (Kakehi et al. 1988). This form of drug resistance (Pgp-MDR) can be

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circumvented in vitro by chemosensitisers that interact with and block the drug-efflux function of Pgp (Kakehi et al. 1988). Clinical investigations using these reverting agents in combination with cytotoxic drugs have been initiated (Mickisch et al. 1990), but the results of these efforts have not been very rewarding so far (Mickisch et al. 1996; Motzer et al. 1995), despite progress in the availability of modern chemosensitisers. These poor results may suggest that other mechanisms of MDR may be operational in RCC. Because RCC specimens display varying levels of Pgp expression (Kanamaru et al. 1989; Volm et al. 1993), Pgp-MDR may be only one of the contributing factors to the MDR of RCC. Other mechanisms of MDR have now been identified. One of these, termed altered-topoisomerase MDR or at-MDR (Beck et al. 1994a; Hochhauser and Harris 1993), is associated with decreased activity of the nuclear enzyme topoisomerase II (topoII). This enzyme can alter the topological state of DNA during cell-cycle activities (Wang 1987). The action of topoII includes cleavage of double-stranded DNA, ATP-dependent passage of a secondary DNA strand through this cleavage site and rejoining of broken DNA (Osheroff et al. 1994). This happens at sites that are exposed to torsional stress of the highly compacted DNA incurred during DNA replication, RNA transcription, and segregation of chromosomes during mitosis. During the breaking and rejoining of double-stranded DNA, topoII and DNA form covalently bound complexes, termed cleavable complexes. The mechanism of action of certain topoII inhibitors, such as the epipodophylotoxins, is to stabilize these cleavable complexes (Liu et al. 1983), thus preventing strand religation and further DNA processing, which eventually will lead to cell death (Beck et al. 1994b; Kamesaki et al. 1993; Pommier 1993). The two mammalian isoforms of topoII, topoIIα (170 kDa) and topoIIβ (180 kDa) (Drake et al. 1987; Tan et al. 1992), have very similar regions, suggesting a comparable mode of action (Chung et al. 1989), but they differ in cell-cycle distribution. Expression of topoIIα varies during cell cycle, whereas topoIIβ appears to be constant (Kimura et al. 1994; Woessner et al. 1990). Altered topoIIα activity, which can be caused by quantitative (Fry et al. 1991; Gudkov et al. 1993; Kim and Beck 1994; Ritke and Yalowich 1993) or qualitative (Danks et al. 1993; Hinds et al. 1991) changes, can result in reduced numbers of drug-stabilized cleavable complexes and is likely to influence cellular sensitivity to topoII inhibitors.

We investigated whether at-MDR plays a role in the chemoresistance of RCC, as previously suggested (Volm et al. 1993). Our aim was to develop a preclinical model for chemotherapeutic drug studies of RCC by establishing a panel of cloned cell lines expressing the at-MDR and/or Pgp-MDR phenotypes via pulsed treatment of previously unselected RCC cell lines with VP-16. The resistance of these newly established RCC sublines against topoII-inhibiting and non-topoisomerase-inhibiting drugs was assessed in relation to alterations in resistance-related gene expression, protein expression and protein activity. In addition, factors that may be responsible for the observed alterations were studied, such as gene mutations in certain regions of interest and methylation within the promoter region of the topoIIα gene.

Materials and methods

Cell culture and conditions

Two non-chemoselected human RCC cell lines, RC2 and RC21, originally established in our laboratory (Grossi et al. 1992) and three resistant sublines were examined. All RCC cell lines were cultured in DMEM-F12 medium supplemented with 7.5% fetal calf serum, 1 E/ml penicillin, 1μg/ml streptomycin and 2 mM glutamine (Gibco BRL Europe, Breda, The Netherlands) at 37°C in a humidified atmosphere supplemented with 5% CO_2. Resistant cell lines were derived from the parental cell lines by selection in increasing concentrations (0.033–10 μg/ml) of etoposide (Bristol-Meyers, Syracuse, USA). Nearly confluent cultures were exposed for 48 h to etoposide in concentrations that killed approximately 90% of the cells. After this treatment cultures were allowed to repopulate and then reexposed to a higher drug concentration for another 48 h. Thus, the etoposide dose was increased stepwise until cultures continued to grow exponentially in the presence of up to 10 μg/ml. The RC21A subline was obtained after 5 months, whereas the RC2E and RC21E lines were both isolated after approximately 8 months. Finally, the sublines were cloned by the limiting-dilution technique and characterized with respect to their resistance to etoposide and to vinblastine (E. Lilly Nederland, Nieuwegein, The Netherlands). The human leukemic CCRF-CEM cell line and its subline CEM/VM-1-5, resistant to teniposide (Danks et al. 1988), served as controls for some of the assays. All cell cultures used for experiments were in logarithmic growth and had been found to be negative for *Mycoplasma*.

Growth inhibition assays

Chemosensitivities of parental cell lines and derived sublines were determined using the microtiter tetrazolium test (Romijn et al. 1988). Exponentially growing cells were detached with trypsin/EDTA (Gibco) and equal numbers of cells were plated with 100 μl medium in 96-well plates (Costar, Cambridge, Mass.). We used separate plates for each cell line and each drug. Plating densities were chosen such that exponential growth was maintained throughout the subsequent 4-day culture period. Drugs were added 24 h after plating in ten different concentrations, dissolved in 100 μl culture medium. Cytotoxic drugs used were etoposide, vinblastine, and doxorubicin (Pharmachemie, The Netherlands). After incubation with cytotoxic drugs for 72 h, 30 μl 5-mg/ml solution of dimethylthiazoldiphenyltetrazolium bromide (Sigma, St Louis, Mo.) in phosphate-buffered saline was added. After 4 h the fluid content of each well was aspirated carefully and the purple formazan precipitate dissolved upon shaking for 3 min in 100 μl 90% dimethylsulfoxide buffered with 0.01 M glycine and 0.01 M NaCl (pH 10.5). Absorbances were measured at 570 nm using a BioRad microplate reader model 450 (BioRad, Hercules, Calif.). The reduction in absorbance at each drug concentration (8 wells) was expressed relative to the untreated controls. IC_{50} values were determined from dose/inhibition curves. Resistance of sublines relative to parental lines was expressed as the ratio of their IC_{50} values (the median inhibitory dose).

Topoisomerase unknotting activity

Isolated nuclei were used to obtain nuclear protein extracts as described before (Danks et al. 1988). Exponentially growing cells (approximately 10^8) were permeabilized by incubation in hypotonic

buffer I (5 mM KH_2PO_4, 2 mM $MgCl_2$, 4 mM dithiothreitol (DTT), 0.1 mM Na_2EDTA, pH 7.0). When almost all cells were stained with trypan blue, they were pelleted at 400 *g* for 5 min and suspended in 2 ml buffer II (buffer I with 0.25 M sucrose). This suspension was layered over 3 ml buffer III (buffer I with 0.6 M sucrose) and centrifuged at 2000 *g* for 20 min. The pellet of isolated nuclei was then incubated in a half-volume of buffer IV (5 mM KH_2PO_4, 4 mM DTT, 1 mM Na_2EDTA, pH 7.0) on ice for 15 min. For 1.0 M salt extracts of nuclear protein, another half-volume of buffer V (40 mM TRIS, 2 M NaCl, 4 mM DTT, 20% glycerol, pH 7.5) was added. After incubation on ice for 30 min, the suspension was centrifuged at 100 000 *g* for 60 min, protein concentration was determined, and aliquots of this solution were stored at −80°C. In all buffers proteinase inhibitors were included.

P4 DNA (knotted phage DNA) was used as a substrate to measure the unknotting activity of topoII in the nuclear extracts, as described previously (Danks et al. 1988). Activity profiles were assessed by electrophoresis of the products in 0.7% agarose, subsequent staining of the gels with ethidium bromide and photographing under UV illumination.

Gene expression and mutation analysis

Total cellular RNA was extracted with the Stat-60 RNA isolation kit (Biotecx Laboratories Inc., Houston, Tex.), according to the manufacturer's directions, and used for single-strand conformational polymorphism (SSCP) analysis of point mutations (Danks et al. 1993) and quantifitation by the reverse transcriptase/polymerase chain reaction (RT-PCR). RNA quantity and quality were checked spectrophotometrically. To generate cDNA from RNA templates, each sample tube included 0.5 μg total RNA; 5 mM $MgCl_2$; 1 mM each dGTP, dATP, dTTP, dCTP; 0.5 unit RNase inhibitor; 2.5 μM random primers; and 1.25 units reverse transcriptase, in a volume of 10 μl. All reagents used for PCR were from Promega (Madison, Wis.). The RT program used was 15 min at 42°C, 5 min at 99°C and 5 min at 5°C. To amplify specific regions from the cDNA, 40 μl was added of the following solution: 2 mM $MgCl_2$, 0.5 μM each primer, 1.25 units Taq polymerase and, if used for SSCP analysis, 1 μCi [^{32}P]dATP. The program to amplify double-stranded DNA was 28 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C. Primer sets used for SSCP were for the ATP-binding sites motif B/DNBS (Danks et al. 1993) of topoIIα: 5′-ATA ATA GAA TCA AGG GAA TTC CCA AAC TCG A-3′ (sense) and 5′-CTT GTA CTG CAG ACC CAC A-3′ (antisense), and for the DNA-binding region adjacent to Tyr-805 of topoIIα: 5′-CAA TTA GCT GGA TCC GTG GCT GAA ATG TC-3′ (sense) and 5′-CAG TAC CGA TTC CTG CAG CAC CAT TTA TC-3′ (antisense). RT-PCR primer sets as described earlier were used for analysis of the expression of the gene for topoII*β* (Tsai-Pflugfelder et al. 1988), *MDR1* (Noonan et al. 1990), and the glyceraldehyde-3-phosphate dehydrogenase gene (Gekeler et al. 1994). Expression of the topoIIα gene was determined using the Tyr-B05 region primer set described above for SSCP analysis. PCR products were separated on 1.75% agarose gels at 150 V for 2 h, stained with ethidium bromide, visualized under UV illumination and photographed using Polaroid 667 film. Products used for SSCP analysis (Danks et al. 1993) were heated to 99°C in formamide for 6 min, placed on ice allowing single DNA strands to retain their three-dimensional conformation, and electrophoresed in a gel composed of 6% acrylamide, 0.12% bisacrylamide, and 10% glycerol at 30 W for 9.5 h. Gels were put onto Whatman 3-mm paper, wrapped in plastic and exposed to X-ray film (Kodak, Rochester, N.Y.).

Immunoblotting

For immunoblotting of topoIIα and topoII*β*, whole-cell protein lysates were prepared as described previously (Danks et al. 1988). After being scraped from culture flasks, approximately 5×10^7 cells were lysed in 1 ml buffer (10 mM TRIS/HCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.8) containing proteolysis inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml soybean trypsin inhibitor, 50 μg/ml leupeptin, 1 μg/ml pepstatin, 20 μg/ml aprotinin) on ice for 20 min. Cells were checked for lysis by trypan blue and were then incubated for 20 min at 37°C after addition of 120 units DNase I (Sigma). The protein amount was quantified (BioRad protein assay) and 1 μl sample buffer (0.125 M TRIS/HCl, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, 0.004% bromophenol blue)/μg protein was added. Samples were boiled for 2 min, and were loaded (50–100 μg protein) onto a 5%–15% gradient sodium dodecyl sulfate/polyacrylamide gel and electrophoresed for 3 h at 25 mA. Proteins were transferred to nitrocellulose (Schleicher & Schüll, Dassel, Germany) with a semi-dry blot apparatus (Milliblot, Millipore, USA) at 2.5 mA/cm^2 gel for 55 min. Membranes were incubated overnight at 4°C with either the anti-topoIIα polyclonal antibody MAC (Friche et al. 1992), or anti-topoII*β* polyclonal antibody (a kind gift of Dr. F. Boege, University of Würzburg, Germany), diluted in blotting milk (0.9% NaCl, 10 mM TRIS/HCl pH 7.5, 0.02% sodium azide, 5% dry milk, 3% IgG-free bovine serum albumin, 0.2% Tween-20). Membranes were then incubated with alkaline-phosphatase-linked goat anti-(rabbit Ig) secondary antibody, and stained with 0.003% 5-bromo-4-chloro-3-indolyl phosphate and 0.006% nitrobluetetrazolium chloride in TRIS buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM TRIS, pH 9.5) until specific bands appeared. For detection of Pgp, cells were solubilized in 20 mM TRIS/HCl (pH 7.4), containing 1 mM $MgCl_2$, 2% v/v Triton X-100, 0.1% w/v SDS, 1% v/v dimethylsulfoxide and proteolysis inhibitors, overnight at 4°C. After centrifugation, the resulting supernatants were filtered (0.45 μm filter) and immediately loaded onto a 7% acrylamide gel. Resolved proteins were transferred to nitrocellulose, which was incubated with the anti-Pgp monoclonal antibody C219. Further incubations were successively performed with biotinylated goat anti-(mouse IgG2a), streptavidin-biotinylated horseradish peroxidase complex, and freshly prepared chemiluminescence Western blotting detection reagent (Boehringer, Mannheim, Germany). Finally, X-ray films were exposed to membranes for 0.2–1 min.

Methylation status of promoter DNA

Genomic DNA was isolated from approximately 2×10^7 cells, and digested as previously described (Loflin et al. 1994). Restriction enzymes *Sty*I and *Xmn*I (New England Biolabs, Beverly, Mass.) were used to obtain the promoter region of the topoIIα gene (Hochhauser et al. 1992). This 623-bp fragment was digested with the restriction enzymes *Hha*I, *Hpa*II and *Msp*I (New England Biolabs). All products were separated on 1.4% agarose gel, transferred to nylon membranes, and hybridized with a ^{32}P-labelled topoIIα-promoter probe (a generous gift of Dr. I.D. Hickson, Oxford, England). X-ray films were exposed to membranes for ±7 days.

Results

Expression of resistance and cross-resistance in etoposide-selected RCC lines

Cells were exposed to chemoresistance-inducing regimens until continuous growth was observed in the presence of 10 μg etoposide/ml. Three sublines thus obtained, RC2E derived from RC2, and RC21A and RC21E, both derived from RC21, were used for further studies. Expression of MDR phenotypes in these sublines was tested by sensitivity assays with etoposide and doxorubicin, both inhibitors of topoII, or vinblastine, which does not inhibit topoII. Dose/response curves for the parental and derived cell lines are shown in Fig. 1. The results demonstrate that all the selected sublines

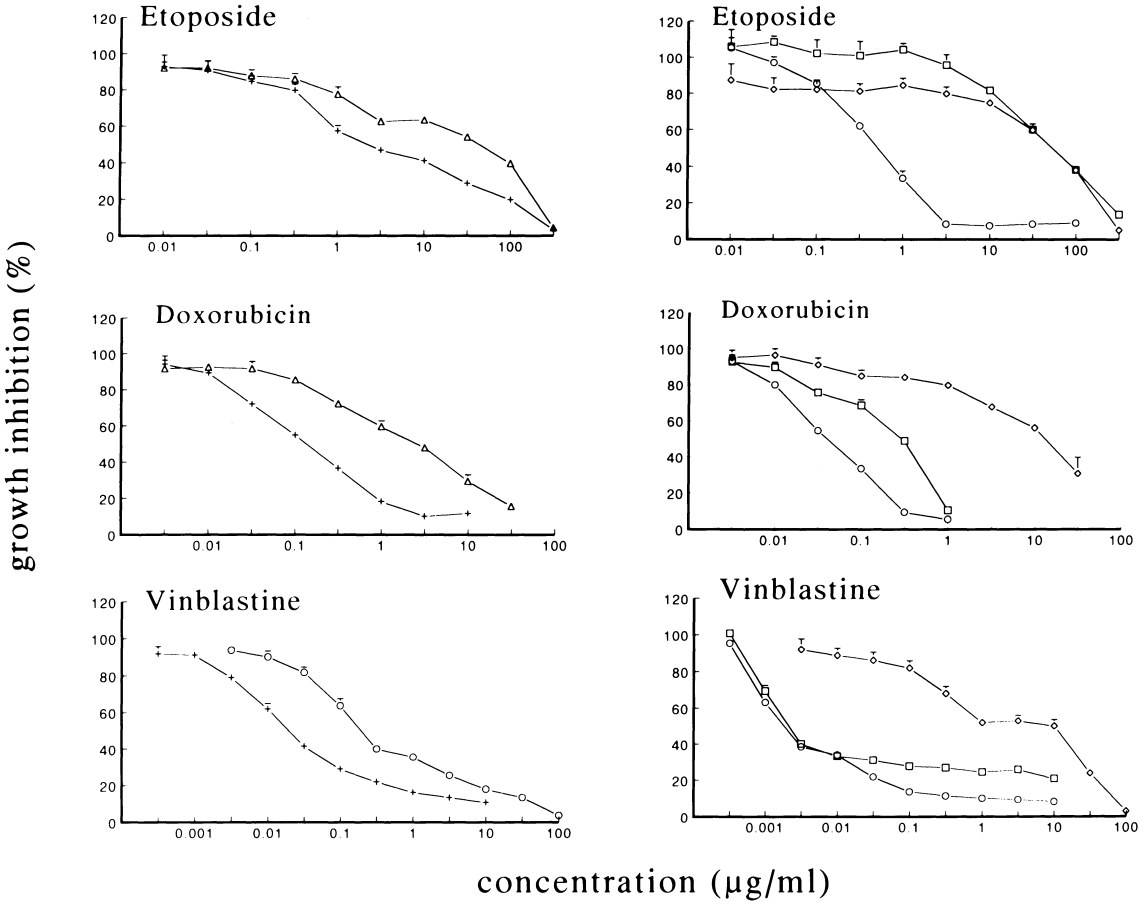

Fig. 1 Cytotoxicity of etoposide, doxorubicin and vinblastine as determined by a microtiter tetrazolium test, measuring growth inhibition, to RC2 (+), RC2E (△) (*left panels*), and RC21 (○), RC21E (□) and RC21A (◇) (*right panels*). Treatment with different drug concentrations extended for twice the doubling-time of the cells. Results are expressed as the mean ± standard deviation of 8 wells of a representative experiment

have a reduced sensitivity to the topoII-inhibiting drugs, as compared to the parental cell lines. The results of several experiments are summarized in Table 1. Resistance to etoposide was increased approximately 30-fold for RC2E, 60-fold/RC21A and 90-fold for RC21E. Enhanced resistance to doxorubicin (approximately 50-, 5-, 400-fold, respectively) was also observed for the same set of cell lines. However, since etoposide and doxorubicin are also known to be potential substrates for Pgp, and RCC can express Pgp, the possible involvement of Pgp was examined as well, by evaluation of the cross-resistance to vinblastine. RC2E and RC21E were indeed strongly cross-resistant to vinblastine (approximately 200- and 3500-fold increase respectively), whereas RC21A appeared to be as sensitive as the parental RC21 cell line (Fig. 1, Table 1).

Table 1 Cross-resistance and doubling times of RC2, RC21 and sub-lines selected for resistance to etoposide. IC_{50} is the 50% inhibitory concentration in a growth-inhibition microtiter tetrazolium test. Values shown are means of three or more separate experiments ±SD. Values were determined by graphical extrapolation of growth curves. The doubling times were defined as the number of hours required for cells in log-phase growth to double in number and were determined graphically from growth curves

	IC_{50} (μg/ml)				
Drug	RC2	RC2E	RC21	RC21E	RC21A
Vinblastine	0.03 ±0.01	6.53 ±6.39	0.004 ±0.002	14.9 ±6.19	0.002 ±0.001
Etoposide	1.68 ±0.87	54.4 ±44.7	0.79 ±0.22	70.2 ±9.03	45 ±17.4
Doxorubicin	0.21 ±0.08	9.59 ±7.53	0.05 ±0.01	19.6 ±5.59	0.31 ±0.22
Doubling time (h)	47	50	37	48	48

The stability of this resistance was checked monthly for more than 1 year. MDR patterns of RC21E and RC21A were stable during this period even without monthly maintenance drug dosages of 1 μg/ml etoposide for 2 days. RC2E lost its resistance 8 months after the last concentration level of etoposide used (10 μg/ml).

Topoisomerase unknotting activity

The ATP-dependent strand-passing activity of topoII in nuclear extracts of the cell lines was measured using P4 DNA as a substrate. P4 DNA was less effectively un-

knotted by extracts of line both cell lines with altered topoIIα gene expression than by the parental cell line. Figure 2 shows separate P4-DNA-unknotting assays of the resistant lines compared to the parental cell line. The amount of nuclear protein required for complete P4 DNA unknotting by the resistant cell lines RC21E and RC21A was double that for RC21. The decreased ability of comparable amounts of nuclear protein to unknot P4 DNA indicates that resistance to etoposide of RC21E and RC21A may be a consequence of altered topoisomerase-associated MDR. Because we saw decreased topoII unknotting activity in the resistant sublines RC21A and RC21E, but not in the resistant line RC2E, we checked the expression of *MDR1*-related genes.

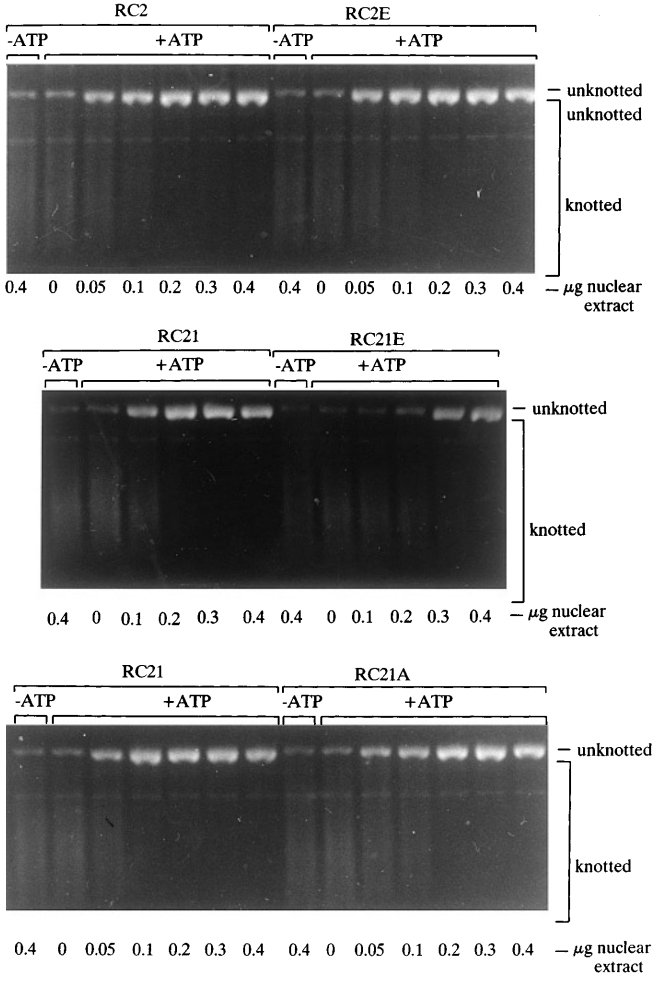

Fig. 2 P4-DNA-unknotting assays comparing RC2E, RC21E and RC21A activities with those of the parental cell lines RC2 or RC21 respectively. Indicated amounts of nuclear protein extracts (1.0 M NaCl) were incubated with P4 DNA with and without ATP for 30 min. Full unknotting, a specific activity of topoisomerase II, is reached when the band of knotted P4 DNA has disappeared. The samples were treated with proteinase K and electrophoresed in a 0.7% agarose gel. Results are representatives of three or more separate experiments

RT-PCR studies

Expression of the resistance-associated genes was examined by RT-PCR, using specific primers. Results are shown in Fig. 3. The level of expression of the "housekeeping" gene for glyceraldehyde-3-phosphate dehydrogenase was used as a control for cDNA quantity. The parental RC2 line and its resistant subline RC2E had the same topoIIα mRNA expression (Fig. 3 upper panel). In contrast, sublines RC21E and RC21A showed a decreased level of topoIIα mRNA, compared to their parental line. RC2E, which expressed resistance to vinblastine, showed a slightly increased *MDR1* mRNA expression compared to RC2 (Fig. 3 lower panel). In contrast to the vinblastine-sensitive lines RC21 and RC21A, the resistant subline RC21E showed *MDR1* expression. Expression signals of the topoIIβ gene were faint, but expression in RC2E and RC21A (Fig. 3 upper panel) appeared to be slightly increased compared to their parental lines.

Immunoblotting

The contents of Pgp, topoIIα and topoIIβ were analyzed by Western blotting of whole-cell protein extracted from the parental cell lines and the derived sublines (Fig. 4). Protein expressions were analyzed for at least three

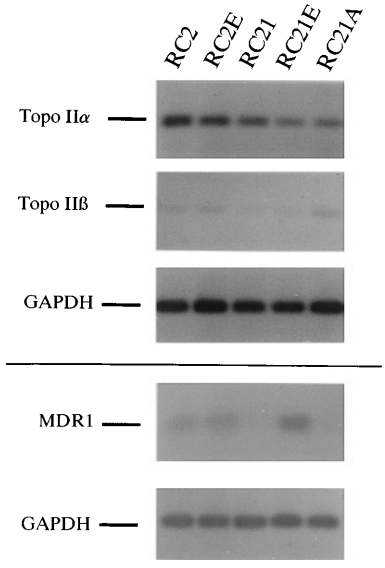

Fig. 3 Analysis of drug-resistance-associated gene expression by reverse transcriptase/polymerase chain reaction (RT-PCR) of RC2, RC2E, RC21, RC21E and RC21A in two separate assays. Sample products were visualised following 1% agarose gel electrophoresis. The signal intensity of the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPH*) amplification products was used as a control for the amount of RNA. *Upper panel* expression of topoisomerase (*Topo*)IIα and topoIIβ mRNA. *Lower panel* expression of *MDR1* mRNA. Results are representative of at least two separate experiments using different RNA isolations

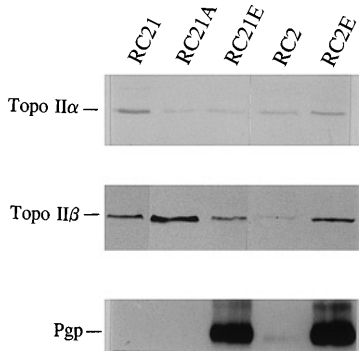

Fig. 4 Immunoblot analysis of topoIIα, topoIIβ and P-glycoprotein (*Pgp*) from RC21, RC21A, RC21E, RC2 and RC2E cells. The expressions in the cell lines demonstrated were obtained in one gel run for each protein indicated. For analysis, cell fractions from each cell line were subjected to SDS-PAGE and the separated proteins were transferred to a nitrocellulose filter; the filters were then prepared as indicated in Materials and methods

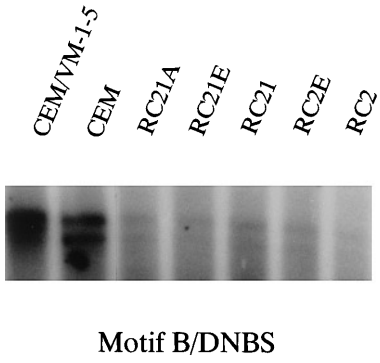

Fig. 5 Autoradiogram of a single-strand conformational polymorphism gel showing migration of PCR products of the motif B/DNBS sequences of topoIIα cDNA generated from RNA of CEM, CEM/VM-1-5, parental and resistant renal cell carcinoma (RCC) cells. Mutated PCR products of CEM/VM-1-5, with a known mutation at nucleotide 1346, migrated more slowly than those from CEM cells (controls, without any mutations). The three bands shown by CEM/VM-1-5 contain both normal and mutated alleles of the complementary single-stranded DNA fragments, but the upper band, containing a normal and a mutant allele, is not separated by the electrophoresis conditions applied. RCC lines do not exhibit point mutations in the motif B/DNBS sequences

different protein extractions of each cell line. The amount of topoIIα protein in subline RC2E, which demonstrated a high level of resistance against both etoposide and vinblastine, was comparable to that in the parental line RC2. Interestingly, both RC21A and RC21E showed a substantial decrease in topoIIα protein expression. The densitometrically determined amount of protein showed a 3-fold reduction in the amount of topoIIα in RC21A and RC21E, compared to RC21. The overexpression of topoIIβ protein in RC2E and RC21A compared to the parental cell lines is in agreement with the RT-PCR findings. While RC21 and RC21A had no visible Pgp expression, sublines RC2E and RC21E, both with decreased sensitivity to vinblastine, had increased Pgp expressions. We can exclude involvement of *MRP1* (Cole et al. 1992) in the MDR phenotype of our RCC cell lines. *MRP1* gene and protein expression was not detectable by either immunocytochemistry (the antibody was a kind gift of R. Scheper, Free University, Amsterdam, The Netherlands) or by RNase protection assay (results, not shown, were obtained in collaboration with K. Nooter, Erasmus University, Rotterdam, The Netherlands).

SSCP analysis

To try to account for the decreased expression of topoIIα protein, we examined by SSCP analysis two regions of the topoIIα gene where, relatively infrequently, point mutations leading to functional aberrations are known to occur (Danks et al. 1993), changing the ATP-binding region (ATP-binding sites: motif B and DNBS) and the DNA-binding region (adjacent to Tyr-805). Mutations in the ATP- and DNA-binding regions, as seen in the CEM/VM-1-5 cells, may result in changed protein configuration and decreased enzymatic activity. Results of the analysis of nucleotides 1323–1534, containing the motif B and DNBS sites, are shown in Fig. 5. No conformational differences were found in the single-stranded PCR products of all of the RCC lines, as they migrated as those of the CEM cell line, which has no mutations. Only the PCR product of CEM/VM-1-5 cDNA showed a third band, which is due to a mutation in this region in one of its alleles. Also, no alterations were found in the ATP-binding region of the parental and resistant RCC cell lines (results not shown).

Methylation status

Since methylation of gene promoter sites can result in gene inactivation (Cedar 1988), we examined the methylation status of the promoter region of the topoIIα gene in RC21E and RC21 cells. RC21A was not analyzed because this is a precursor of RC21E, unlikely to exhibit changes that are not seen in the latter line. Restriction enzyme analysis using *Hha*I and *Hpa*II (cutting at unmethylated sites only) and *Msp*I (cutting at the same sites as *Hpa*II regardless of the methylation status) was applied to identify possible resistance-related differences (Harker et al. 1995; Jones and Buckley 1990). Figure 6 shows that identical patterns were obtained for RC21 and RC21E topoIIα-gene-promoter DNA with *Hha*I (94-bp and 529-bp fragments), *Hpa*II (43-bp, 241-bp and 338-bp fragments) or with *Msp*I (the same fragments as with *Hpa*II). We conclude that hypermethylation of the promoter region of the topoIIα gene does not seem to play an important role in the decreased transcription level of topoIIα RNA.

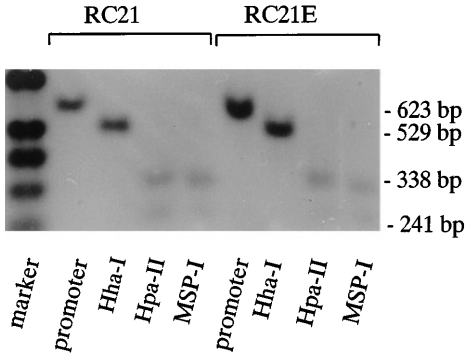

Fig. 6 Methylation analysis of the DNA of the topoIIα gene promoter (623 bp) from RC21 and RC21E cells. *Hha*I (digesting unmethylated GCGC sites), *Hpa*II (digesting unmethylated CCGG sites) and *Msp*1 (digesting the same CCGG sites, methylated and unmethylated) produced identical fragments of 94 bp and 529 bp or 241 bp and 338 bp, respectively, from both RC21 and RC21E (94-bp fragment not visible)

Discussion

The nuclear enzymes topoI and topoII are the targets of many known drugs that stabilize complexes of broken DNA strands and protein (Pommier 1993). Most of these drugs stabilize covalent complexes of topoII and broken DNA strands that are formed during specific phases of the cell cycle, leading to apoptotic cell death (Walker et al. 1991) by mechanisms that are incompletely understood. Alterations of the level of topoII activity, which can be caused by a variety of mechanisms such as topoIIα gene mutations (Danks et al. 1993; Hinds et al. 1991; Nitiss et al. 1993), post-translational modifications (Devore et al. 1992; Takano et al. 1991), or a reduced amount of the enzyme (Friche et al. 1991; Gudkov et al. 1993; Liu 1989), will therefore modulate the effectiveness of topoII-specific drugs. If fewer complexes are stabilized, cells will be less vulnerable to cytotoxic drugs and may become resistant. To date, the majority of chemoresistance studies in RCC have focused on the expression of Pgp, the *MDR1* gene product, as a cause of resistance to a broad range of antineoplastic drugs. Since there is a considerable variability of Pgp expression in RCC, the unrewarding results of chemotherapeutic treatment for RCC can not exclusively be explained by Pgp-mediated resistance. In the present study, we found that at-MDR resistance, associated with a reduced expression of the topoIIα gene and protein, may contribute to the MDR phenotype in RCC.

Cloned drug-resistant sublines RC2E, RC21A and RC21E were generated from the parental, non-chemoselected, human RCC cell lines RC2 and RC21 by stepwise exposure of cell cultures to increasing concentrations of etoposide. We showed that RC2E overexpressed Pgp, that RC21A exhibited a decreased amount of topoIIα, and that RC21E developed both forms of resistance. As a consequence, improvement of the chemotherapeutic treatment of RCC may require drugs that are substrates for neither Pgp-mediated MDR nor non-Pgp-mediated MDR, such as at-MDR. Our panel of cell lines, therefore, offers a new tissue-culture model that can be used to identify novel chemotherapeutic agents, including topoIIα-interactive drugs, and to investigate the mechanisms of at-MDR resistance in RCC. It may be possible that other mechanisms, which not have been investigated yet, such as glutathione content or the activity of glutathione transferase, may be part of the MDR phenotype as well. Involvement of multidrug-resistance-associated protein 1 (MRP1), a cell-membrane-bound protein, is excluded in these cell lines.

We observed a reduced growth rate and altered growth pattern or cell cultures of RCC cells expressing reduced amounts of topoIIα protein. In contrast to the parental RC21 cell line, the sublines continue to proliferate after reaching confluence, resulting in the formation of multilayer colonies. Reduced growth rate has also been described for other cancer cell lines (Danks et al. 1988), which may complicate the interpretation of the growth-inhibiting effects of drugs. However, the reduced cytotoxic effects of topoII-inhibiting drugs will likely lead to quantifiable tumor expansion. Another consequence of the reduced growth rate can be that the cloning procedure of the resistant sublines may not have resulted in the selection of the most resistant cells. The cloning technique, which selects colonies formed from a single cell, will favor faster-growing cells. Thus, for reliable comparisons, growth-adjusted IC_{50} values should be used for the determination of cytotoxicity.

Occasionally, an indirectly correlated expression of the topoI and topoIIα genes is seen in cells resistant to topoisomerase inhibitors, which might be interpreted as a compensatory mechanism (Woessner et al. 1992). Others found a direct correlation between topoIIα and topoIIβ gene expression (Brown et al. 1995; Kaufmann et al. 1994). We observed increased topoIIβ protein expression in RC21A cells and reduced topoIIα, which would be in accordance with the idea of compensation. It remains unclear why RC2E expresses an increased amount of topoIIβ protein, without reduced expression of topoIIα. Thus, up-regulation of topoIIβ protein might be regarded as an optional mechanism of compensation for a decrease in topoIIα protein. However, topoIIβ gene expression does not seem to affect cellular P4-DNA-unknotting activity, since RC2E, with increased topoIIβ expression, showed unaffected unknotting activity, compared to the parental RC2. In addition, RC21A showed decreased unknotting activity, although it expresses increased topoIIβ protein, compared to parental RC21.

Decreased amounts of topoIIα, seen in RC21A and RC21E, are matched by decreased mRNA expression, suggesting that these lines express the at-MDR phenotype. Also, we detected no point mutations in the regions of the gene encoding the motif B/DNBS and Tyr-805, as analyzed with SSCP. These regions represent areas in the topoIIα gene sequences in which some point mutations

were found in other malignancies (Danks et al. 1993). The occurrence of mutations in other parts of the gene cannot be excluded, however. On the other hand, test conditions used to run the gels are claimed to be highly efficient for the specified regions. Nevertheless, it appears that reduced activity of topoII in the drug-resistant sublines, as assessed by P4-DNA-unknotting activity, is due to decreased expression of the topoIIα gene.

In our initial investigations for a possible cause of the decreased topoIIα gene expression, we measured transcription activity of the topoIIα gene, in part by comparing the methylation status of the promoter region of the topoIIα gene in the RC21 and RC21E cell lines; we found no differences between these cell lines (Fig. 6), and the topoIIα gene promoter was hypomethylated. Methylation of RC21A was not investigated since RC21E was established by continuous exposure of RC21A to etoposide, when decrease of topoIIα gene expression had already taken place. It is, therefore, highly unlikely that changes in RC21A could be detected that would not found in RC21E also. Our results suggest that a decrease in topoIIα protein in RC21E cells is likely due to other mechanisms of gene regulation. One possible mechanism for such gene regulation is transactivation of the Sp1 and Sp3 proteins. These proteins might activate or inhibit the promoter region of the topoIIα gene respectively, as has been described in other cell lines (Kubo et al. 1995).

RCC cells, which are almost always resistant to a broad range of antineoplastic drugs, are well known to express *MDR1* and Pgp. The established RCC cell lines overexpress *MDR1* and are highly resistant to vinblastine, but it has become clear that at-MDR likely contributes to the drug-resistance profile in RCC. The observed resistance to topoII inhibitors of at-MDR RCC cell lines appears to be related to a decrease in topoIIα gene and protein expression. Expression of the topoIIα gene in clinical specimens, when primary cultures are used, has already indicated a possible involvement of at-MDR in RCC (Volm et al. 1993). However, a direct relationship between expression and resistance was not investigated in that study. The immortal and cloned cell lines described in this paper showed such a relationship in RCC cells. These lines, with their defined resistance profiles and expression patterns of resistance markers, offer a model suitable for extensive in vitro investigation of drug development in RCC, and for elucidating the mechanism of topoII activity and regulation of gene expression. Future studies with this model should yield information on the drug-resistance profiles of new chemotherapeutic agents and may contribute to improved treatment of clinical RCC.

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