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Topoisomerase expression in cancer cell lines and clinical samples

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Abstract. Topoisomerase II activity has been previously associated with chemosensitivity to cytotoxic agents in cell lines made resistant to drugs in vitro. Examination of unselected cancer cell lines, however, shows a relatively poor correlation between topoisomerase II content and intrinsic chemoresistance. Studies of topoisomerase II expression in clinical materials from human tumor biopsies also demonstrate a poor relationship with the response of the cancers to induction chemotherapy. A major problem with assessing topoisomerase II activity in clinical materials is the marked heterogeneity of the enzyme among the cells and the associated high proportion of tumor cells which are not traversing the cell cycle. While the activation of oncogenes may disregulate topoisomerase II expression in some experimental systems, there is currently no evidence that enzyme activity is disconnected from cell cycling in clinical cancer specimens. Novel techniques of topoisomerase II measurement may permit more accurate correlation of enzyme activity with clinical chemosensitivity.

Key words: Topoisomerase inhibitors – Drug resistance – Leukemia

Introduction

DNA topoisomerases are among the most important targets for cancer chemotherapy, and numerous studies have examined the relationship between topoisomerase I and II expression and relative resistance to cytotoxic agents. Multiple human cell lines have been grown in the presence of increasing concentrations of topoisomerase II inhibitors [1]. It is very well established that cell lines made resistant to these drugs in vitro may have decreased topoisomerase II content or activity [2] or may, in some cases, have mutations in the topoisomerase II gene [3]. This review does not address these lines but instead focusses on studies of unselected cell lines, which examine the link between topoisomerase activity and intrinsic chemoresistance.

There are relatively few studies of topoisomerase II levels in malignant cells taken directly from patients. These investigations, however, are critically important for an understanding of why topoisomerase-directed drugs do or do not work in the clinical setting. This review focuses particularly on the differences in topoisomerase expression between cancer cell lines and clinical samples and the implications of these differences on chemoresistance. The principles of topoisomerase expression in clinical materials are summarized as they are currently understood, and several newer techniques that make easier the daunting task of analyzing topoisomerase content and activity in clinical specimens are reviewed.

Regulation of topoisomerase activity

The expression of DNA topoisomerase II is closely regulated by the state of cellular proliferation in nontransformed cells, and cells that are in a low proliferating state experience little damage when exposed to topoisomerase II-directed chemotherapy. Quiescent Chinese hamster ovary (CHO) cells are less sensitive than log-phase CHO cells to etoposide [4]. This loss of sensitivity is associated with a decrease in topoisomerase enzyme activity in nuclear extracts of the quiescent cells. A reduction in enzyme content correlates in most cell lines with the ability of the cells to accumulate during quiescent periods with a G_0-G_1 content of DNA. Sensitivity to the DNA-cleavage effects of etoposide in dividing and nondividing cells correlates well

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with enzyme content. The low DNA topoisomerase II content in immunoblots of quiescent CHO cells suggests that the loss of enzyme activity in CHO cells is a function of a reduction in content rather than posttranslational modifications in the enzyme.

Quiescent human lymphoblastic CCRF cells also have lower topoisomerase II content than do actively proliferating cultures, but the difference is smaller than that observed in CHO cells. In contrast, log- and plateau-phase cultures of mouse leukemia L1210 cells exhibit similar topoisomerase II content. As had been observed with CHO cells, both CCRF and L1210 cells in the plateau phase are more resistant to the cytotoxic effects of etoposide than those actively dividing, even though the L1210 cells had equivalent DNA damage as observed under the two growth conditions. This finding indicates that topoisomerase II enzyme content is proliferation-dependent in some but not all cells and suggests that although enzyme content may be important for drug resistance in some cell types, other factors can decrease the sensitivity of the cell to cleavable-complex formation as well.

The cytotoxic effects of topoisomerase II inhibitors require at least 3 events: [1] intracellular drug accumulation, [2] stabilization of topoisomerase II-DNA adducts, and [3] conversion of these adducts into lethal damage. Treatment of HL-60 human leukemia cells with dichloro-*B*-D-ribofuranosylbenzimidazole, an inhibitor of RNA synthesis, diminishes the cytotoxicity of etoposide or 4-(9-acrydinylamino)-methanesulfon-*m*-aristide (m-AMSA) to a greater degree than treatment with aphidicolin, an inhibitor of DNA synthesis [5]. The formation of topoisomerase II-DNA adducts is unaffected by either agent. These results suggest that ongoing nucleic acid synthesis, particularly RNA synthesis, plays a role in the conversion of topoisomerase II-DNA adducts into cytotoxic damage.

Topoisomerase expression in cancer cell lines

Several attempts have been made to correlate the activity of topoisomerase II with sensitivity to chemotherapy in panels of unselected cancer cell lines. Fry et al. [6] compared levels of topoisomerase II in three testicular cancer and three bladder cancer cell lines. The testicular cancer lines were more sensitive than the bladder cancer lines to m-AMSA, doxorubicin, and etoposide. The frequency of DNA strand breaks induced in the testicular cancer lines by m-AMSA was 1.5- to 13-fold higher than that observed in the bladder cell lines. Western blotting showed generally higher topoisomerase II levels in testicular than in bladder nuclear extracts. Topoisomerase II protein expression broadly correlated with drug-induced strand breakage in both protein extracts and whole cells. However, in spite of a 2- to 20-fold increase in sensitivity to various topoisomerase II inhibitors, the testicular cancer line 833 K had a less than 2-fold higher level of topoisomerase II protein than did the bladder line RT4, suggesting that additional factors must contribute to the extreme chemosensitivity of testicular cancer cells.

Giaccone et al. [7] studied drug sensitivity in relation to topoisomerase II gene expression and activity in eight human lung-cancer cell lines. The cytotoxicity of doxorubicin, etoposide, teniposide, cisplatin, camptothecin, and 5-fluorouracil (5-FU) were measured. The lung cancer cell lines demonstrated a common pattern of multidrug resistance. The correlation was best for the topoisomerase II-targeted agents and cisplatin, less strong for camptothecin, and weak for 5-FU. A 10-fold difference in topoisomerase II gene expression was found in the cell lines, which could not be explained by differences in the doubling time or cell-cycle distribution of the lines. Topoisomerase II expression correlated with the cell sensitivity to etoposide, teniposide, doxorubicin and even cisplatin. However, one non-small-cell lung-cancer cell line that was found to have low levels of topoisomerase II was highly sensitive to all drugs. The levels of topoisomerase I did not correlate with sensitivity to any drug.

Kasahara and co-workers [8] examined topoisomerase II expression in relation to chemosensitivity in three non-small-cell lung-cancer and four small-cell lung-cancer (SCLC) cell lines. SCLC are generally more sensitive to anticancer agents than are non-SCLC. Topoisomerase II activity was assayed in this study by the decatenation of kinetoplast DNA to free minicircles, and topoisomerase II content was determined by immunoblot analysis of nuclear extracts. The SCLC lines were more sensitive to doxorubicin and etoposide than were the non-SCLC lines. Etoposide uptake in SCLC cells was significantly higher than that in non-SCLC cells, but the small difference noted could not account for the variation in sensitivity. The topoisomerase II activity and immunoblot content of SCLC nuclear proteins were reproducibly 2-fold higher than those of the non-SCLC lines and corresponded to the relative sensitivity of the lines to doxorubicin and etoposide.

Gazdar et al. [9] established cell lines from 27 patients with SCLC prior to initial treatment with etoposide and cisplatin. In vitro cytotoxicity assays with etoposide on these lines correlated surprisingly well with the response and survival of the patients. From these lines, Doyle et al. [10] chose six that were sensitive and seven that were resistant to etoposide for further evaluation. Low levels of P-glycoprotein were found in some small-cell lines by Western blotting but did not correlate with in vitro resistance to etoposide. The relative expression of topoisomerase II RNA and protein in the 13 lines correlated well as determined by Western and Northern blotting. The levels of topoisomerase II varied 8-fold among the SCLC lines and did not correlate well with the wide range of in vitro sensitivity to etoposide. There was also a poor correlation between topoisomerase II levels and the decatenating activity of topoisomerase II from nuclear extracts. In particular, two cell lines with high levels of topoisomerase II expression were resistant to etoposide in vitro and had weak decatenating activity relative to the other SCLC cell lines as determined by a kinetoplast DNA (kDNA) assay. The authors concluded that factors other than the amount of detectable topoisomerase II protein must be important in determining the sensitivity of unselected SCLC cells to etoposide.

Houlbrook and co-workers [11] examined the chemosensitivity of a panel of six breast-cancer and six lung-cancer cell lines to a number of topoisomerase II inhibitors

(m-AMSA, etoposide, and doxorubicin) after 4 days of continuous exposure to drug in tetrazolium-dye (MTT) assays. Statistical analysis indicated a correlation in sensitivity between the lines to various topoisomerase II inhibitors, although the degree of this correlation was variable. Topoisomerase II levels were measured by scanning Western blots prepared from nuclear extracts derived from exponentially growing cells. Topoisomerase II was detected using antibodies T2K1, T2K2, and CRB, which had been raised to peptide sequences of the α form of topoisomerase II. The sequence used to raise T2K2 has homology with topoisomerase IIβ and detects two bands, but the other two sequences are α-specific and detect only the 170-kDa band. No obvious correlation was seen between topoisomerase II levels and drug sensitivity in the cell lines.

Immunocytochemical techniques have recently been employed to examine the localization of topoisomerase II after exposure to a specific inhibitor. Wolverton et al. [12] used polyclonal antisera to examine the nuclear distribution of topoisomerase II in cytospin preparations of drug-sensitive CEM lymphoblastic leukemia cells and teniposide (VM-26)-resistant CEM sublines. The nuclear distribution of topoisomerase II in monolayer cultures of a human rhabdomycosarcoma (Rh30) cell line was also examined. In the absence of drug, focal patchy staining of nuclear topoisomerase II was noted in all cell lines. However, treatment of CEM and Rh30 cells with VM-26 increased the intensity and homogeneity of nuclear topoisomerase II staining in a subpopulation of cells. This effect was proportional to the amount of either drug and occurred rapidly. The VM-26-mediated alteration in topoisomerase II staining intensity and distribution was attenuated in proportion to the degree of VM-26 resistance in the CEM/VM-1 and CEM/VM1-5 sublines. These results appear to be related to the ability of the drug to stabilize DNA topoisomerase covalent complexes in intact cells.

In conclusion, topoisomerase studies in unselected human cell lines have demonstrated activity and chemosensitivity roughly corresponding to characteristics of the tumors from which the cell lines were derived. Discrepancies between topoisomerase activity and in vitro chemoresistance have been noted in several models, and the differences in topoisomerase function appear inadequate to explain fully the differences in resistance to topoisomerase inhibitors.

In vivo analysis of topoisomerase function in animal xenografts

Several investigators have used cultured tumor cells implanted in animals to approximate more closely the clinical setting in cellular growth regulation and drug delivery. Yamauchi et al. [13] examined the cytotoxicity of camptothecin and etoposide in relation to topoisomerase I and II levels in human gastrointestinal cancer xenografts in nude mice. A camptothecin-resistant colon-cancer xenograft from cell line CC-2-NU/CPT showed decreased topoisomerase I activity as compared with a xenograft from the parental cell line. Xenografts from two colon-cancer and two stomach-cancer cell lines were examined for sensitivity to camptothecin or etoposide. Camptothecin was effective in three of these lines in which topoisomerase I activity was readily detectable. Protein levels of topoisomerase II were low in each line and the xenografts were uniformly resistant to etoposide. This study did not address the possible role of P-glycoprotein in preventing cytotoxicity. Etoposide, but not camptothecin, is a substrate for P-glycoprotein, which is known to be heavily expressed in colon-cancer cells.

Tilchen et al. [14] used fetal mouse squamous-cancer cell lines ASB and LC12 to implant BALB/c mice subcutaneously with 30- to 60-mg fragments. After 7 days, animals bearing tumors were treated with varying doses of camptothecin or etoposide. There was no response to camptothecin at any level in either tumor. The use of etoposide resulted in a 1.7-$\log_{10}$ kill in the ASB model and in a 4.3-$\log_{10}$ kill in the LC12 model. Tumor from control animals was harvested and sequestered into an enriched aneuploid population. Semiquantitative reverse transcriptase/polymerase chain reaction assays in these aneuploid fractions revealed barely detectable levels of topoisomerase I mRNA but easily detectable amounts of topoisomerase II mRNA in both lines. Topoisomerase II expression was greater in LC12 tumors than in ASB tumors. The authors concluded that the RNA levels of the target enzymes correlated with the in vivo sensitivity to the agents.

Comparison of topoisomerase II activity in clinical samples versus cell lines

Exposure of chronic lymphocytic leukemia cells or normal lymphocytes to m-AMSA has been found to cause at least 50-fold less DNA-cleavage than is seen with the L1210 cell line [15]. Immunoblotting of topoisomerase II in chronic lymphocytic leukemia cells or normal human lymphocytes reveals less than 1% of the levels seen in exponentially growing L1210 cells. These results suggest that low levels of topoisomerase II could contribute to drug resistance found in human malignancies with a large compartment of nonproliferating cells.

Edwards and co-workers [16] examined topoisomerase II content and drug-induced DNA cleavage in freshly obtained human leukemia cells. The human T-lymphoblast line CCRF-CEM was more than 100-fold more sensitive to DNA cleavage by etoposide than were the cells of the 13 leukemic patients examined. Another leukemia cell line (HL-60) and a lymphoblastoid line (RPMI-7666) were somewhat less sensitive than the CCRF-CEM line but were nonetheless 10-fold more sensitive than the acute myeloblastic leukemia (AML) blast samples studied. The relative insensitivity of the freshly obtained cells could not be accounted for by differences with respect to drug uptake but was associated with markedly reduced topoisomerase II content as assayed by immunoblotting using a mouse polyclonal serum. Heterogeneity was observed in the sensitivity of patients cells with respect to both drug-induced DNA-cleavage and enzyme content.

Kaufmann et al. [17] found that etoposide also induced fewer strand breaks in AML samples than in HL-60 cells. Western blotting revealed diminished levels of both topoisomerase II isoforms in the clinical samples. Levels of

topoisomerase IIα and IIβ were lower in 46/47 clinical samples than in the human AML cell lines used as controls.

Giaccone et al. [18] found that 9/56 non-SCLC biopsies had undetectable topoisomerase II levels and that the other samples had greatly reduced topoisomerase II levels as compared with those of lung-cancer cell lines. In 16 samples of normal lung tissue obtained from the patients, no topoisomerase II activity could be demonstrated.

Topoisomerase II studies in human tumor specimens

Recent investigations have attempted to correlate topoisomerase II steady-state levels or activity from fresh tumor samples with the response of the patients to topoisomerase-directed chemotherapy. In the study mentioned above, Giaccone et al. [18] found no correlation between topoisomerase II levels and the clinical response or other patients characteristics in biopsies from 56 patients with non-SCLC. Doyle et al. [19] correlated topoisomerase II expression in fresh leukemic blast cells with the success of induction therapy with daunorubicin and cytarabine (ara-C) in previously untreated AML patients. Steady-state levels of topoisomerase II RNA from 20 patient samples were quantitated using Northern blotting and ^{32}P detection of labeled-probe DNA with a Betascope. Western blotting was performed on blast cell lysates from 44 patients using specific antisera to topoisomerase II, and the topoisomerase bands were quantitated by densitometry. Standard lanes using the leukemic cell line HL-60 were run on each Northern or Western blot. In addition, each blot was rehybridized with an actin probe or antisera to control for variation in loading. A 20-fold range of topoisomerase II expression in both the RNA and protein blots was found, with no correlation with FAB subtype being established. There was no correlation between topoisomerase II expression and the clinical response, although this might easily have been obscured by the small sample size and the contribution of cytarabine to the leukemic induction regimen.

Kaufmann et al. [17] found that colony-forming assays showed that the dose of etoposide, daunorubicin, or m-AMSA required to diminish leukemic colony formation by 90% (LD_{90}) in a large number of AML marrow aspirates varied over a >20-fold range between different pretreatment marrow specimens. Although two of the three agents utilized in this treatment were topoisomerase II-directed, there was no correlation between topoisomerase II content and the clinical response or the remission duration. Although this may be explained as an effect of cytarabine, no correlation was noted between topoisomerase II content and the sensitivity of AML blasts to daunorubicin or m-AMSA in vitro.

Topoisomerase I studies in human tumor samples

There is currently a very limited published record of topoisomerase I activity in fresh tumor specimens in relation to chemosensitivity. Adjei and Kaufmann studied topotecan (TPT) in patients with refractory leukemia (S. Kaufmann, personal communication). TPT, a camptothecin analogue that inhibits topoisomerase I, was given as a 5-day continuous infusion to patients with relapsed and refractory leukemias. A total of 17 patients received 31 courses at doses ranging from 0.7 to 2.7 mg/m^2 per day. Significant diminution of circulating blasts occurred in all cases at all dose levels. One complete remission in a patient with chronic myelogenous leukemia in blast crisis and one partial remission in an AML patient were documented. Clonogenic assays revealed that the LD_{90} for TPT during a 5-day period of exposure in vitro ranged from 6 to 22 n*M*, and pharmacologic data from 12 patients (18 cases) revealed that these levels were readily achievable and could be maintained for 5 days. Steady-state concentrations of total TPT were 8.5–37.8 n*M*. Western blotting of 15 patients' samples revealed a wide range of topoisomerase I levels in the leukemic blasts. However, there was no correlation between topoisomerase I levels and the antileukemic effect of TPT.

Correlation of topoisomerase expression with cell cycling in clinical specimens

Although topoisomerase expression clearly correlates with cell cycling and the rate of proliferation in nontransformed cell lines, the correlation is less clearly demonstrable in neoplastic cell lines and tumor biopsies. Previous reports have suggested that topoisomerase II levels diminish when nontransformed tissue-culture cells enter the G_0 phase but remain high when L1210 mouse leukemia cells or HeLa human cervical carcinoma cells reach the plateau phase. These observations led to the suggestion that pathways linking topoisomerase II levels and cell-cycle traverse might be deranged in neoplastic cells.

Van der Zee and co-workers [20] did not observe any correlation of topoisomerase I or II activity with the mitotic index in malignant ovarian cancer specimens. Fogelsong et al. [21] performed immunocytochemical measurements of the DNA topoisomerase II α and β isoforms on a variety of tumor samples and attempted to correlate topoisomerase expression with that of proliferating cell nuclear antigen (PCNA). The immunoreactivities of topoisomerase IIα and PCNA were similarly elevated in 24/37 specimens, whereas only 13/37 specimens yielded high levels of both PCNA and topoisomerase IIβ. Both isozymes of topoisomerase II were elevated in only 9/37 tumor samples. Six tumor specimens exhibited low levels of both topoisomerase II isoforms but elevated amounts of PCNA. Two specimens demonstrated an elevation in topoisomerase IIα but not in PCNA or topoisomerase IIβ.

The relationship between the percentage of topoisomerase IIα-positive AML cells and the percentage of cells in the S phase has been examined by immunohistochemistry studies in 11 AML samples, each containing >80% blasts [17]. The frequency of topoisomerase IIα-positive cells tended to increase as the number of cycling cells increased, but this trend did not reach statistical significance in this small series. The proportion of topoisomerase II-positive cells was often larger than the proportion of S-phase cells,

but topoisomerase II is known to be present in other phases of the cell cycle.

The observations in AML blasts do not support a model of deranged cell-cycle expression of topoisomerase II. In human AML specimens, topoisomerase II is diminished in or absent from the majority of cells. The blasts that contain detectable levels of topoisomerase II appear to be a subset of the cells that are traversing the cell cycle. In short, there is no evidence that the linkage between cell-cycle traverse and topoisomerase II expression is abnormal in AML.

Recent studies suggest that the cytotoxicity of topoisomerase II-directed agents results from the conversion of reversible drug-stabilized topoisomerase II-DNA adducts into cytotoxic lesions through an interaction with advancing replication or transcription complexes. Murine leukemia cell lines contain abundant levels of topoisomerase II; 35% of these cells are in the S phase and virtually all are traversing the cell cycle in vivo. In human AML, the percentage of cells containing topoisomerase II and the percentage of cells traversing the cell cycle are smaller, leading to a decreased efficacy of topoisomerase II inhibitors in human AML. These findings suggest that caution is needed in the interpretation of cytotoxicity studies involving topoisomerase poisons tested in model cell lines.

Correlation of topoisomerase I and II expression in clinical neoplasms before and after drug treatment

Several recent studies have examined the linkage between topoisomerase I and II expression in fresh malignant materials and the relative expression of topoisomerase II isoforms. Van der Zee et al. [20] found that topoisomerase II activity correlated with topoisomerase I activity in malignant ovarian cancers. A range of 8- and 16-fold differences in topoisomerase I and II activity, respectively, were found in the malignant tumors. Similarly, in AML blast cells, Kaufmann et al. [17] found that only rare patient samples had a preponderance of one topoisomerase II isoform over the other. Generally, samples that were high in one isoform were high in the other.

Cancer cell lines frequently down-modulate topoisomerase II expression in response to the selective pressure of in vitro exposure to topoisomerase II inhibitors. It is therefore of considerable interest to examine whether similar down-modulation of topoisomerase II occurs in tumors exposed to drugs in vivo. Van der Zee and co-workers [20] found that topoisomerase II activity was decreased in malignant ovarian cancers after cisplatin/cytoxan treatment relative to the untreated samples, whereas no difference was found between median levels of topoisomerase I activity in treated versus untreated samples. Although neither of the cytotoxic agents used in this study were topoisomerase II-directed, it should be borne in mind that the results obtained by Giaccone et al. [18] and other investigators [9] link resistance to topoisomerase II inhibitors with resistance to cisplatin. In contrast, Kaufmann et al. [17] found in AML marrow aspirates that there was a strong correlation between topoisomerase II levels determined at diagnosis and those measured at relapse. This correlation was found for both topoisomerase II isoforms.

Variation in topoisomerase II expression by tumor type

Topoisomerase II activity clearly appears to vary with different malignant histologies. Whereas topoisomerase II activity is not detectable in chronic lymphocytic leukemia (CLL) cells, it can be readily detected in cells obtained from patients with diffuse histiocytic, nodular poorly differentiated, and nodular mixed lymphomas as well as in Burkitt's lymphoma, acute lymphoblastic leukemia (ALL), and CLL with prolymphocytic transformation [15]. In contrast, DNA topoisomerase I is detectable in CLL cells and normal lymphocytes as well as in cells of the other malignancies mentioned above.

By Western blotting, topoisomerase II polypeptide can similarly be easily seen in samples of aggressive lymphoma and ALL. However, etoposide-induced strand breaks and topoisomerase II levels are markedly diminished in AML as compared with AML cell lines.

Van der Zee et al. [20] performed immunohistochemistry studies of topoisomerase I and II activity in benign and malignant ovarian tumors. The same specimens were also examined for topoisomerase II activity as measured by relaxation of supercoiled plasmid pBR322 DNA and decatenation of kinetoplast DNA. Median levels of topoisomerase I and II were found to be elevated in malignant ovarian tumors as compared with benign ones.

In an immunocytochemical analysis of 37 specimens from a variety of tumor types, Fogelsong et al. [21] found that the prostate samples tested had uniformly low expression of topoisomerase IIα and all three hepatomas tested had low expression of both topoisomerase isoforms. Carcinomas of the colon, stomach, and lung as well as melanomas, sarcomas, and lymphomas were high in both topoisomerase IIα and IIβ as well as in PCNA expression. Only 1/37 tumors gave low reactivity to all three antigens, the pattern most often observed for most normal tissues.

Heterogeneity of topoisomerase II expression

Kaufmann et al. [17] used immunoperoxidase staining to demonstrate marked cell-to-cell heterogeneity in the content of topoisomerase IIa, with most AML cells lacking detectable topoisomerase IIa. This heterogeneity implies that a low signal on a Western blot may not reflect a low topoisomerase II content in each cell of the population. Marrow mononuclear cells from 40 newly diagnosed AML patients were stained with affinity-purified anti-topoisomerase IIα antibodies. In proliferating AML cell lines, every cell stained for topoisomerase IIα. In contrast, expression of topoisomerase IIα in the clinical samples was heterogeneous, with a strong signal being detected in some blasts and no signal being seen in others. Among the 20 stained marrows that contained $>80\%$ blasts, the percentage of topoisomerase IIα-positive cells varied from $<1\%$ to 40%.

Topoisomerase II mRNA or protein content accurately reflects the content of individual cells only in genetically and phenotypically homogeneous tissue-culture cell lines, where topoisomerase II content varies within fairly narrow confines in actively cycling cells. Staining of topoisom-

erase IIα in AML clinical samples revealed marked cell-to-cell heterogeneity which may help explain the lack of correlation frequently seen between topoisomerase II levels and in vitro and in vivo drug sensitivity.

Western blotting or alkaline elution provides a measure of the "average" topoisomerase II content in a large tumor cell population. Because topoisomerase II is heterogeneously expressed, this average topoisomerase II content may not necessarily reflect the topoisomerase II levels in a subset of cells that are potentially clonogenic in vivo.

Modulation of topoisomerase II content with induced cell cycling

Etoposide's potency in normal resting lymphocytes resembles that observed in circulating leukemia cells [16]. However, following mitogenesis with phytohemagglutinin and interleukin 2 (IL-2), proliferating lymphocytes become as sensitive to etoposide as cultured cell lines with regard to DNA-cleavage. This effect is accompanied by an increase in topoisomerase II content. Data support the hypothesis that topoisomerase II content may be an important determinant of cell sensitivity to certain drugs. Efforts to stimulate topoisomerase II content in leukemia, in a setting of increased cell cycling, might improve the therapeutic efficacy of these drugs.

Kaufmann et al. [17] examined marrow samples from newly diagnosed AML patients who had been treated with granulocyte/macrophage colony-stimulating factor (GM-CSF) for 3 days prior to chemotherapy. Paired samples on day 1 (before GM-CSF) and day 4 (prior to cytotoxic chemotherapy) were stained for topoisomerase IIα. The number of positive cells counted on day 4 increased to $>150\%$ of the day-1 values in 6/14 patients (43%). When the analysis was restricted to marrow samples containing $>80\%$ blasts, the number of topoisomerase IIα-positive cells counted on day 4 increased to $>150\%$ of the day-1 values in 3/4 cases. Bromodeoxyuridine (BUdR) incorporation studies revealed that the increase in topoisomerase IIα occurred concomitantly with an increase in the percentage of S-phase cells to $>150\%$ of control values. The one patient who failed to increase his topoisomerase IIα percentage also did not change his S phase fraction, consistent with the view that the heterogeneity of topoisomerase IIα expression reflects differences in passage through the cell cycle.

The clinical significance of increased topoisomerase II expression in marrow specimens from GM-CSF-treated AML patients is unclear. Towatari and co-workers [22] reported that administration of G-CSF to G-CSF-dependent human leukemia cell lines in vitro resulted in increased topoisomerase II levels and increased etoposide sensitivity. Treatment of two AML patients with GM-CSF in Kaufmanns et al.'s series [17] resulted in concomitant increases in the percentage of blasts traversing the cell cycle and the percentage of cells containing detectable levels of topoisomerase IIα. These findings strengthen the relationship between cell-cycle traverse and topoisomerase II expression in human AML. On the other hand, the marked heterogeneity of topoisomerase II expression among cells from each AML aspirate makes it difficult to determine whether topoisomerase II expression is changing in the clonogenic cells. It is currently unclear whether the administration of GM-CSF prior to topoisomerase II-directed therapy will result in any therapeutic benefit in leukemia or other malignancies.

Factors leading to a lack of correlation between topoisomerase II activity and cytotoxicity in human neoplasms

The correlation between topoisomerase II content and sensitivity to topoisomerase II-directed agents observed in model systems was not detectable in studies of AML blasts [17]. These results suggest that cellular heterogeneity may have obscured any underlying relationship between topoisomerase II content and drug sensitivity. Clonogenic assays have previously shown correlations between sensitivity to 4-hydroperoxycyclophosphamide in vitro and the clinical response to cytoxan-containing regimens in adult leukemia [23]. Similar correlations between in vitro sensitivity to cytarabine and clinical responses to AML induction chemotherapy have also been noted. The latter correlation suggests that cytarabine might have been the dominant agent in Kaufmann et al.'s regimen, although it was paired with two topoisomerase II inhibitors. Other investigators have seen correlations between anthracycline sensitivity in vitro and responses in AML.

Recent studies suggest that the activation of oncogenes in some tumors may modulate topoisomerase activity. Woessner et al. [24] studied the activity and content of the 170- and 180-kDa topoisomerase II isoforms in relation to cellular transformation and growth state in parental and *ras*-transformed NIH-3T3 cells. Total topoisomerase II activity as determined by unknotting of P4 DNA was higher in the *ras*-transformed cells. Total topoisomerase II levels measured by immunoblotting were also dependent on transformation. The proportion of the 170-kDa isoform was higher in *ras*-transformed cells and less dependent on the growth state of the cells. The topoisomerase II activity in extracts of *ras*-transformed cells was more sensitive to inhibition by teniposide and merbarone, drugs that selectively inhibit the 170-kDa form. The susceptibility of certain tumors to killing by topoisomerase II-directed drugs may be due to a higher proportion of the 170-kDa enzyme as well as a higher level of total topoisomerase II activity.

Cunningham et al. [25] examined cells from family members with the Li-Fraumeni syndrome, which is an inherited susceptibility to a wide variety of neoplasms that has been shown to have an intriguing association with an aberrant *c-raf-1* gene. Since topoisomerases can be modulated by serine/threonine kinases similar to *raf*-encoded proteins, the investigators studied *v-raf/c-myc* and *EJ-ras*-transformed NIH/3T3 cells. Each of the oncogene-transformed cells demonstrated a similar perturbation of a spermidine- and adenosine triphosphate (ATP)-dependent DNA catenation activity typical of topoisomerase II. Relaxation of DNA supercoiling and other topoisomerase I assays were not abnormal in the transformed cells. Cytotoxicity assays and evaluation of the influence of topo-

isomerase II inhibitors on DNA/protein complex formation corroborated the existence of a qualitative topoisomerase II defect in the transfectants and in cells from Li-Fraumeni family members. Although the contention that a qualitative topoisomerase II abnormality may be associated with altered sensitivity to chemotherapy is speculative, these findings may be relevant to tumors in Li-Fraumeni family members as well as to human tumors associated with *raf*- and *ras*-type oncogenes.

Alterations in the accumulation or nuclear transport of cytotoxic drugs in tumors could lead to discrepancies between topoisomerase II activity and cell killing by topoisomerase-directed agents. Concomitant cytarabine use can explain the lack of correlation between topoisomerase II levels and the clinical response to combination chemotherapy in AML, but it does not explain the lack of correlation between topoisomerase II levels and drug sensitivity to daunorubicin or m-AMSA in vitro. To investigate this discrepancy and to address the problem of multidrug resistance in relapsed AML, a number of investigators have examined the expression of P-glycoprotein and other putative drug transporters in AML cells.

Ross et al. [26] used functional assays to examine the effects of P-glycoprotein modulators on daunorubicin uptake in blast cell samples from 49 newly diagnosed AML patients. Cyclosporin A caused a greater than 20% enhancement of daunorubicin accumulation in over 50% of the patient samples as well as a greater than 40% enhancement of daunorubicin cell killing in vitro. The largest differences in modulation of daunorubicin accumulation (approximately 2-fold) appear inadequate to explain the range of drug sensitivity among the AML specimens. Surprisingly, Western blotting for P-glycoprotein demonstrated that only three patients samples had detectable P-glycoprotein, and two of these samples were obtained from patients who had gone into complete remission. Kaufmann et al. [17] also detected very few P-glycoprotein-positive samples from untreated AML patients. Sensitive assays such as the reverse transcriptase/polymerase chain reaction can detect much higher frequencies of low-level P-glycoprotein in AML blasts, but this expression has not been shown to correlate with the clinical response of AML patients to induction therapy [27].

Other membrane transporters, modulated by cyclosporin A, may be functionally significant in AML cells. A reverse transcriptase/polymerase chain assay for the recently described *MRP* gene demonstrated very low expression of this gene in 21 AML blast samples (D. Ross, personal communication). However, Doyle et al. [28] found that expression of a recently described 95-kDa surface protein (P-95), overexpressed in multidrug-resistant cancer cells, was significantly correlated with decreased daunorubicin accumulation in AML blast cells.

Mutations in topoisomerase II genes, especially in neoplasms relapsing after chemotherapy, could certainly explain some instances of altered chemoresistance. Kaufmann et al. [17] found no evidence for mutated topoisomerase II in AML aspirates from resistant patients. Since patients in the study received m-AMSA and daunorubicin, these investigators initially searched for a G-A mutation at position 1493 of the topoisomerase IIα gene that had been noted to occur independently in two intercalator-resistant HL-60 cell lines. Genomic DNA from the marrow of 23 AML patients was amplified by polymerase chain reaction using primers that encompassed the nucleotide of interest and was then digested with MseI, a restriction enzyme that cleaves the amplified DNA if the mutation is present. This analysis was performed on 7 samples obtained at the time of diagnosis from patients who subsequently failed to achieve a complete remission, on 13 samples obtained at the time of disease recurrence from refractory and relapsed patients, and on 5 samples with an m-AMSA LD_{90} of >10 μM. There was no evidence of the G-A mutation in any of these samples.

RNA from six AML samples with an m-AMSA LD_{90} of >10 μM and readily detectable levels of topoisomerase II was subjected to a reverse transcriptase/polymerase chain reaction using primers that spanned the ATP B and gyrase domains of topoisomerase IIα and IIβ. These domains encompass all currently identified mutations resulting in the resistant phenotype. Sequencing of the cDNA obtained from these clinical samples revealed only wild-type topoisomerase IIα and IIβ sequences. There was no evidence of the mutations observed in resistant cell lines.

Danks et al. [29] recently described the lack of mutations in the ATP B and gyrase domains of topoisomerase IIα in relapsed ALL patients, also suggesting that topoisomerase II mutations probably did not obscure an underlying relationship between topoisomerase II levels and drug sensitivity.

Other genetic alterations of the topoisomerases could be postulated to affect their expression or functional activity. Keith et al. [30] examined amplification of the topoisomerase IIα gene in human breast cancer biopsies. The topoisomerase IIα gene is located in very close proximity to the *ERBB2* gene on chromosome 17q. Of 50 tumors analyzed, 6 had amplifications of *ERBB2*, of which 3 had co-amplification of topoisomerase IIα. Tumors with amplified topoisomerase IIα were also found to overexpress high levels of the protein.

Genetic alterations have also been noted at topoisomerase I loci of human solid tumors [30]. Restriction-fragment-length polymorphism (RFLP) analysis performed for topoisomerase I demonstrated allelic loss in 2/17 breast-cancer biopsies and 4/25 lung cancers. This allelic loss could potentially be paired to mutations of the other allele, leading to a resistant phenotype.

Novel techniques to monitor topoisomerase expression in clinical samples

The logistical and technical difficulties of obtaining and analyzing clinical tumor specimens for topoisomerase expression and function has inhibited such correlative studies until the recent past. Several new techniques have been developed, however, which may increase the ease and precision of topoisomerase measurement in future studies.

Competitive topoisomerase II reverse transcriptase/polymerase chain reactions have been developed that allow quantification of topoisomerase II mRNA in tumor samples. The topoisomerase II cDNA is synthesized from cel-

lular RNA using topoisomerase II-specific primers. Using RNA from doxorubicin-resistant and parental human lung-cancer cell lines, a good correlation has been demonstrated between the polymerase chain reaction and decatenation or Western blot assays [31]. Whether the polymerase chain reaction will have any advantages in correlating topoisomerase II expression with the in vitro cytotoxicity of tumor materials or the clinical response of cancer patients is yet to be determined.

Immunohistochemistry studies will assume an increasing role in measurement of topoisomerase expression, especially as the problems of cellular heterogeneity become more apparent. The use of automated systems to process multiple slide specimens simultaneously under identical conditions will allow concomitant measurement of topoisomerases to be performed in conjunction with that of other immunohistochemistry antigens [21].

Ellis and co-workers [32] have recently developed a simple filter-binding assay that can measure drug-induced DNA-protein cross-links in leukemia cells obtained directly from patients more easily than sodium dodecyl sulfate (SDS)/KCl precipitation or alkaline elution assays. HL-60 cells or leukemia aspirates can be incubated with topotecan, etoposide, or m-AMSA; lysed with SDS; and applied to nitrocellulose filters. DNA is retained on the filter only if it is covalently bound to protein. The amount of DNA retained can be quantified by hybridization to the *alu* sequences of DNA that are distributed ubiquitously in the human genome. The investigators used radiolabeled cells to compare the filter-binding assay directly with the SDS/KCl precipitation assay for the detection of etoposide- or m-AMSA-induced DNA-protein cross-links in HL-60 and m-AMSA-resistant HL-60/AMSA cells. Both the SDS/KCl precipitation assay and the filter-binding assay detected m-AMSA- and etoposide-induced DNA-protein cross-links in HL-60 and HL-60/AMSA cells and detected a greater frequency of m-AMSA-induced DNA-protein cross-links in HL-60 cells than in HL-60/AMSA cells. The filter-binding assay has also been used to detect DNA-protein cross-links in freshly isolated leukemia cells exposed to topotecan in vitro. The ratios of DNA retention found for topotecan-treated versus untreated cells from leukemia patients ranged from 1.8 to 11.5. This new filter-binding technique may be useful for predicting the sensitivity of individual patients' tumors to drugs that inhibit type I or type II DNA topoisomerases.

Conclusion

The lack of correlation between topoisomerase levels or activity and both in vitro cytotoxicity and clinical responses in several important tumor types indicates that we currently have an inadequate understanding of topoisomerase-mediated cytotoxicity in the clinical setting. Principles of topoisomerase function and interaction with drugs learned from tumor cell lines cannot necessarily be applied to human neoplasms. The heterogeneity of topoisomerase activity and cell cycling is far greater in tumors than in cell lines adapted to in vitro growth. Topoisomerase mutations, which are being detected with increasing frequency in drug-resistant cell lines, have not appeared thus far to play a major role in clinical resistance to topoisomerase poisons.

The relative expression and activity of topoisomerase I and II and of the topoisomerase II isoforms may vary depending on the histology of the tumor being studied. The activation of oncogenes in certain human tumors may also potentially modulate the activity of topoisomerase proteins. Although these problems are daunting, the careful analysis of large numbers of clinical samples has now begun to elucidate the principles of topoisomerase function and drug interaction in a clinically important setting.

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