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Topoisomerase I gene expression and cell sensitivity to camptothecin in human cell lines of different tumor types

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Topoisomerase I (topo I) gene expression and cell sensitivity to camptothecin were investigated in seven human cancer cell lines not selected *in vitro* for drug resistance. The cell lines were of different tumor origin, and included two ovarian carcinomas (A2780 and IGROV-1), a cervix squamous cell carcinoma (A431), an osteosarcoma (U2-OS), a glioblastoma (GBM) and two different clones of a malignant melanoma (665/2/60 and 665/2/21). Topo I gene expression was evaluated by Northern blotting analysis and cell sensitivity to camptothecin was determined using the colony-forming assay after a 1 h exposure to the drug. A wide range of drug sensitivity levels was found among the examined cell lines. Cell doubling times and distribution in cell cycle phases were not correlated with camptothecin cytotoxicity. In particular, the percent of untreated cells in S phase was not predictive of the drug sensitivity. No correlation was found between level of topo I gene expression and cell response to camptothecin. These results indicate that the level of topo I expression is not the only critical determinant of cell sensitivity to camptothecin in unselected human cancer cell lines. Therefore, topo I gene expression may not be a useful predictive parameter of tumor response.

Key words: Camptothecin, topoisomerase I.

Introduction

Mammalian DNA topoisomerases are nuclear enzymes involved in crucial functions of DNA such as transcription, replication, recombination and chromosome condensation. They are of great interest in the pharmacology of antitumor drugs since they are recognized as the primary targets of several chemotherapeutic agents.[1–3] In particular, the antitumor activity of camptothecin has been ascribed to a direct effect on DNA topoisomerase I (topo I) function. The drug stabilizes a cleavable enzyme–DNA complex, which can be detected as DNA cleavage products upon sodium dodecyl sulfate (SDS) treatment.[2] Alterations of topo I have been described in camptothecin-resistant cell lines. Decreased expression of the enzyme has been detected in HT29/CPT and St-4/CPT cell lines,[4] and camptothecin-resistant forms of topo I have been isolated from drug-resistant cells.[5–9] However, many of these studies utilized drug-resistant cell lines which had been *in vitro* selected for camptothecin resistance. Thus the role of topo I in the sensitivity/resistance of unselected cells remains to be defined.

In this study, we examined topo I gene expression in seven human cell lines not selected *in vitro* for drug resistance. The results indicate that the cellular level of topo I is not the only determinant of camptothecin activity in unselected human tumor cells of different origin.

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Materials and methods

Drugs

Camptothecin was purchased from Sigma (St Louis, MO), dissolved in dimethyl sulfoxide at 10 mM and diluted in sterile water immediately before use.

Cell lines and growth conditions

The seven human cancer cell lines used in the study included: A2780, an ovarian carcinoma obtained from Dr RF Ozols (National Cancer Institute, Bethesda, MD); A431, a cervical carcinoma (ATCC CRL 1555); IGROV-1, an ovarian carcinoma, kindly supplied by Dr Benard (Institut Gustave Roussy, Villejuif, France); U2-OS, an osteosarcoma cell line

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(ATCC HTB 96); GBM, a glioblastoma cell line, kindly obtained from Dr Boiardi (Istituto Neurologico Besta, Milan, Italy), and 665/2/21 and 665/2/60 cells, two clones derived from a subcutaneous metastasis of a human melanoma, kindly provided by Dr A Anichini (Istituto Nazionale Tumori, Milan, Italy). A2780, A431, IGROV-1, GMB cell lines, and 2/21 and 2/60 clones were cultured in RPMI 1640 containing 10% (v/v) fetal calf serum (FCS); U2-OS cells were grown in McCoy's 5A medium with 10% (v/v) FCS. For the growth curves, cells from maintenance cultures were counted and inoculated into 6-well plates. Daily, duplicate samples were harvested and counted with a Coulter Counter (PBI Electronics, Luton, UK) and the cell number was averaged for each interval. The doubling times were calculated in the logarithmic phase of growth.

Cell distribution in cell cycle phases and DNA index

Exponentially growing cells were harvested and fixed in 70% ethanol, incubated with phosphate buffered saline (PBS) containing RNase A (40 μg/ml) for 15 min on ice and subsequently stained with propidium iodide (20 μg/ml) in PBS for 15 min. Stained cells (10^4) were analyzed by flow cytometry using an Epics C Instrument (Coulter Electronics, Hialeah, FL). The DNA index was calculated by dividing the channel intensity at the G_0–G_1 peak of the cell line tested by that obtained from normal human lymphocytes.

Drug sensitivity assay

Cell survival was assessed by the colony-forming assay. Briefly, exponentially growing cells were exposed to camptothecin for 1 h and then trypsinized, seeded in triplicate samples, and allowed to develop colonies; 7–14 days later, colonies of 30 or more cells were counted after fixing in methanol and staining with crystal violet. The IC_{50} was defined as the drug concentration which reduced colony formation to 50% of values obtained for untreated control cells.

Northern blotting analysis

Total RNA was purified from exponentially growing cells by cell lysis with guanidine isothiocianate and centrifugation in a cesium chloride gradient.[10] About 20 μg of total RNA was electrophoresed in a formaldehyde-containing 1% agarose gel and transferred onto a nylon membrane. A 0.7 kb human topo I cDNA fragment was purified from the plasmid pGEM-4-DI kindly provided by Dr L Liu (Baltimore, MD). A 1.15 kb fragment of mouse β-actin cDNA was derived from plasmid pAL41.[11] DNA probes were ^{32}P-labeled with a random primer kit (Amersham, Little Chalfont, UK). Prehybridization was carried out for at least 4 h at 42°C in 50% formamid, 5 × SSC, 5 × Denhardt's solution, 0.2% SDS and 50 μg denatured salmon sperm. Hybridization was for 16–18 h at 42°C in the same buffer containing 10% dextran sulfate and ^{32}P-labeled DNA probes. Final washes of filters were at 50°C for 20 min in 0.5 × SSC and 0.1% SDS. The expression level was evaluated by densitometric analysis of autoradiograms and compared with the expression level of the control β-actin gene.

Results

Biological characteristics of the tumor cell lines

Seven human tumor cell lines of different type (Table 1) were chosen since they expressed a range of *in vitro* sensitivities to conventional cytotoxic agents. In particular, IGROV-1 and A2780 cell lines were established from ovarian carcinomas,[12,13] the A431 cell line from a cervix squamous cell carcinoma,[14] the GBM cell line from a glioblastoma,[15] U2-OS cell line from an osteosarcoma,[16] and 665/2/21 and 665/2/60 cells from a malignant melanoma.[17] The tumors of origin had not been treated with chemotherapeutic agents at the time of biopsy. However, the patient with the glioblastoma had been exposed to cranial irradiation (radiotherapy 60 Gy) and locoregional immunotherapy with IL-2 and LAK cells.

Among these cell lines, doubling times ranged from 14 ± 0.5 to 73 ± 7.0 h, with the GBM cell line exhibiting the longest one. Cytofluorimetric analysis revealed a large range in ploidy values (1.0–2.2). An analysis of cell distributions through the cell cycle showed that A2780 and GBM cells were characterized by a smaller fraction of cells in the S phase than the other lines. No relation was noted between doubling times and cell cycle phase distributions.

Table 1. Biological characteristics of the studied human cancer cell lines

Cell line	Tumor type	Doubling time[a] (h)	DNA index[b]	Cell cycle phase distribution[c]		
				G_0–G_1	S	G_2–M
A2780	ovarian carcinoma	21 ± 1.0	1.3	65 ± 7.5	15 ± 1.8	20 ± 6.1
IGROV-1	ovarian carcinoma	22 ± 1.0	1.0	46 ± 3.6	21 ± 3.6	33 ± 1.1
A431	cervix squamous cell carcinoma	14 ± 0.5	1.5	51 ± 10.8	23 ± 4.6	26 ± 6.3
U2-OS	osteogenic sarcoma	25 ± 0.5	2.0	54 ± 2.5	20 ± 1.0	26 ± 3.0
GBM	multiform glioblastoma	73 ± 7.0	1.8	63 ± 3.5	16 ± 0.1	21 ± 3.5
665/2/21	malignant melanoma	33 ± 4.0	1.7	55 ± 1.5	19 ± 1.7	26 ± 0.6
665/2/60	malignant melanoma	31 ± 3.0	2.2	51 ± 2.7	31 ± 3.3	18 ± 1.2

[a] Calculated in exponentially growing cells. Values are the mean ± SD of at least three independent experiments.
[b] Relative value to human lymphocytes determined by cytofluorimetric analysis.
[c] Calculated in exponential phase of growth. Values represent the percent of cells in each phase compared with the total cells and are the mean ± SD of two to four independent experiments.

Topo I gene expression and camptothecin cytotoxicity

Sensitivity to camptothecin in these seven cell lines was determined by the colony-forming assay following a 1 h drug exposure. The IC_{50} values were calculated from linear regression analysis of individual experiments and are reported in Table 2. Marked differences in cytotoxicity were observed among the cell lines, with 665/2/21 cells being the most resistant to camptothecin. The range of response varied by 1.5 log as judged by IC_{50} values.

DNA topo I gene expression was determined by Northern blotting analysis (Figure 1A and B). A single 4 kb transcript was detected in all cell lines, in agreement with the reported size of the human topo I message.[18] Gene expression levels were quantified by densitometry of autoradiograms and normalized relative to the expression of a control gene (β-actin). Among the seven cell lines, topo I showed a 7-fold variation, with the 2/21 clone having the highest level, and GBM and U2-OS lines the lowest level of expression.

Figure 2 shows the relationship between cell sensitivity to camptothecin and topo I gene expression. No correlation was noted between cell response to camptothecin and expression of the primary target of the drug.

Table 2. Camptothecin cytotoxicity on the studied human cancer cell lines

Cell line	IC_{50} (μM)[a]
A2780	0.041 ± 0.017
U2-OS	0.135 ± 0.043
A431	0.30 ± 0.038
GBM	0.34 ± 0.10
665/2/60	0.40 ± 0.10
IGROV-1	0.941 ± 0.33
665/2/21	2.20 ± 0.20

[a] Cytotoxicity as assessed by colony-forming assay on cells exposed for 1 h to the drug. Values represent the mean ± SD of two to four independent experiments.

Discussion

Several lines of evidence indicate that topo I is the primary cellular target of camptothecins.[19] The broad antitumor activity of these drugs observed in preclinical studies has been related to a significant expression of the target enzyme in malignant tissues.[20] A direct correlation has been found between cytotoxicity of camptothecin analogs and their ability to inhibit purified enzyme *in vitro*.[21] Thus, the level of topo I might be a critical determinant of camptothecin cytotoxicity.

In this study, using human cell lines of different tumor types and characterized by a different pattern of response to conventional cytotoxic agents, no correlation was found between the level of topo I expression and cell sensitivity to camptothecin. Comparable levels of topo I were detected in five cell lines, including two lines (IGROV-1 and melanoma 2/21) relatively resistant to the cytotoxic action of camptothecin following a short-time

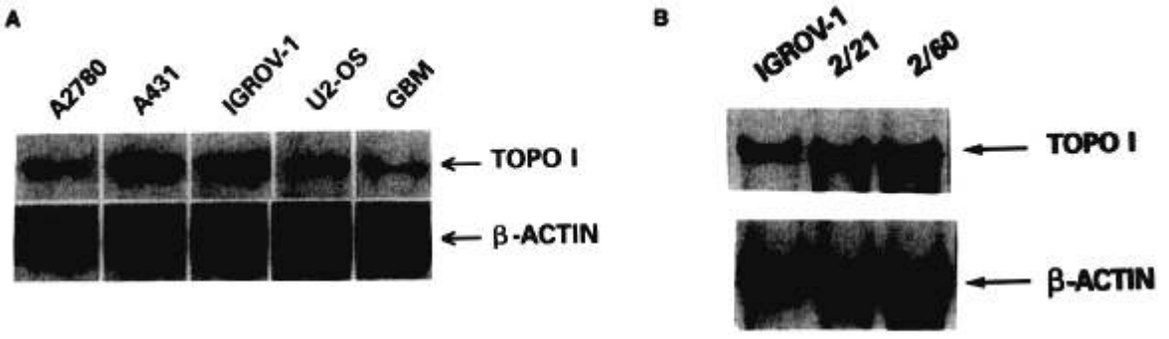

Figure 1. Northern blotting analysis of topo I gene expression in human cancer cell lines (A and B). Total RNA (20 μg) was electrophoresed in a formaldehyde-containing 1% agarose gel, transferred to a nylon membrane and hybridized with a ^{32}P-labeled human topo I cDNA probe (pGEM-4-DI).[18] The filters were then stripped and re-hybridized with a mouse β-actin probe derived from the plasmid pAL41.[11]

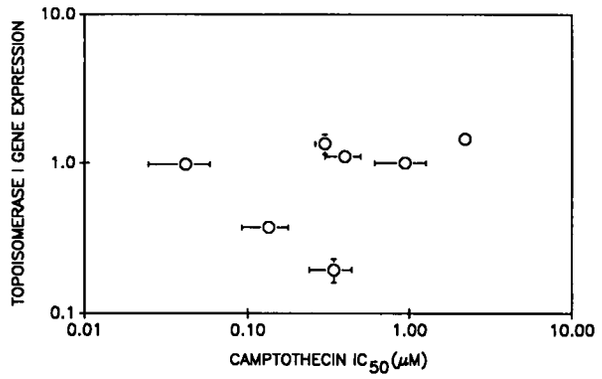

Figure 2. Relationship between topo I expression and camptothecin cytotoxicity. The values for gene expression levels are expressed relative to that of IGROV-1 cells which was set equal to 1. IC_{50} values of camptothecin are from Table 2.

exposure. Two cell lines (U2-OS and GBM) expressed a low level of topo I gene and an intermediate sensitivity to camptothecin. Apparently, the range of cell responses to the drug is not related to growth characteristics. Indeed, no relation has been found between chemosensitivity and proliferation rate or cell cycle phase distributions of the studied cell lines. Slowly proliferating tumor cells may be less sensitive to conventional cytotoxic agents.[22] A similar behavior is also expected for camptothecin action, since involvement of DNA replication seems essential in the multistep process of drug cytotoxicity.[23] Indeed, camptothecin is specifically cytotoxic for S phase cells,[23,24] although cellular levels of topo I are relatively independent of cell cycle phases.[25] The results presented in this work support the view that cell susceptibility to camptothecin is not simply related to the expression of the primary target and the proliferation rate. Indeed, cell lines with comparable topo I expression (A2780, A431, IGROV-1, 665/2/60 and 665/2/21) are characterized by similar growth characteristics (Table 1), but they exhibited a quite variable response to drug treatment. On the other hand, the slowly growing GBM cell line, which expressed the topo I at the lowest level among the examined cells, is moderately sensitive to the drug. It is possible that a correlation between camptothecin cytotoxicity and topo I expression is complicated by multiple cell differences related to tumor origin. However, two clones established from the same melanoma cell line exhibited a markedly different susceptibility to the drug, suggesting cell-specific factors of susceptibility.

Although the pattern of cell response to other DNA damaging agents with a different mechanism of action (e.g. doxorubicin and cisplatin) is not superimposable on that of camptothecin, it is interesting to note that the most sensitive (A2780) and the most resistant (2/21) cell lines are common to all these drugs (not shown). This observation is reminiscent of a previous observation on human lung cancer cells indicating a common pattern of multidrug sensitivity.[26] Camptothecin is involved in this phenomenon.

The results presented in this work are consistent with the view that the presence of topo I is necessary but not sufficient for the cytotoxic effects of camptothecin. Although in this work the extent of protein-associated single-strand DNA breaks have not been measured, it is possible that DNA damage is produced by drug interaction with enzyme function at different genomic sites with variable cyto-

toxic consequences in cell systems with a comparable enzyme expression.

In conclusion, this study raises concerns as to the possibility of using the topo I expression level as a predictive indicator of human tumor response to drug treatment.

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References

1. D'Arpa P, Liu LF. Topoisomerase-targeting antitumor drugs. *Biochim Biophys Acta* 1989; **989**: 163–77.
2. Pommier Y, Kohn KW. Topoisomerase II inhibition by anticancer intercalators and demethylepipodophyllotoxins. In Glazer RI, ed. *Developments in cancer chemotherapy.* Boca Raton, FL: CRC Press 1989: 175.
3. Zunino F, Capranico G. Anthracycline antibiotics as DNA topoisomerase II poisons. *Anti-Cancer Drug Des* 1990; **5**: 307–17.
4. Sugimoto Y, Tsukahara S, Oh-hara T, *et al.* Decreased expression of DNA topoisomerase I in camptothecin-resistant tumor cell lines as determined by a monoclonal antibody. *Cancer Res* 1990; **50**: 6925–30.
5. Andoh T, Ishii K, Suzuki Y, *et al.* Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. *Biochemistry* 1987; **84**: 5565–9.
6. Kjeldsen E, Bonven BJ, Andoh T, *et al.* Characterization of a camptothecin-resistant human DNA topoisomerase I. *J Biol Chem* 1988; **263**: 3912–6.
7. Gupta RS, Gupta R, Eng B, *et al.* Camptothecin-resistant mutants of chinese hamster ovary cells containing a resistant form of topoisomerase I. *Cancer Res* 1988; **48**: 6404–10.
8. Tamura H, Kohchi C, Yamada R, *et al.* Molecular cloning of a cDNA of a camptothecin-resistant human DNA topoisomerase I cDNA and identification of mutation sites. *Nucleic Acids Res* 1991; **19**: 69–75.
9. Tanizawa A, Bertrand R, Kohlhagen G, *et al.* Cloning of chinese hamster DNA topoisomerase I cDNA and identification of a single point mutation responsible for camptothecin resistance. *J Biol Chem* 1993; **268**: 25463–68.
10. Davis LG, Dibner MD, Battey JF. *Basic methods in molecular biology.* Amsterdam: Elsevier/North Holland 1988.
11. Alonso LS, Minty A, Bourlet Y, *et al.* Comparison of three actin coding sequences in the mouse: evolutionary relationships between the actin genes of warm-blooded vertebrates. *J Mol Evol* 1986; **23**: 11–22.
12. Benard J, De Silva J, De Blois MC, *et al.* Characterization of a human ovarian adenocarcinoma line, IGROV1, in tissue culture and in nude mice. *Cancer Res* 1985; **45**: 4970–9.
13. Eva A, Robbins KC, Andersen PR, *et al.* Cellular genes analogous to retroviral *onc* genes are transcribed in human tumour cells. *Nature* 1992; **295**: 116–9.
14. Giard DJ, Aaronson SA, Todaro GJ, *et al. In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J Natl Cancer Inst* 1973; **51**: 1417–23.
15. Perego P, Boiardi A, Carenini N, *et al.* Characterization of an established human malignant glioblastoma cell line (GBM) and its response to conventional drugs. *J Cancer Res Clin Oncol* 1994; **120**: 585-92.
16. Ponten J, Saksela E. Two established *in vitro* cell lines from human mesenchymal tumours. *Int J Cancer* 1967; **2**: 434–47.
17. Supino R, Prosperi E, Formelli F, *et al.* Characterization of a doxorubicin-resistant murine melanoma line: studies on cross-resistance and its circumvention. *Br J Cancer* 1986; **54**: 33–42.
18. Juan CC, Hwang J, Liu AA, *et al.* Human DNA topoisomerase is encoded by a single copy gene that maps to chromosome region 20 q12-13.2. *Proc Natl Acad Sci USA* 1988; **85**: 8910–3.
19. Pometsil M. Camptothecins: from bench research to hospital wards. *Cancer Res* 1994; **54**: 1431–9.
20. Giovanella BC, Stehlin JS, Wall ME, *et al.* DNA topoisomerase I-targeted chemotherapy of human colon cancer in xenografts. *Science* 1989; **246**: 1046–8.
21. Hsiang YW, Liu LF, Wall ME, *et al.* DNA topoisomerase I-mediated DNA cleavage and cytotoxicity of camptothecin analogues. *Cancer Res* 1989; **49**: 4385–9.
22. Goldie JH, Coldman AJ. The genetic origin of drug resistance in neoplasms: implications for systemic therapy. *Cancer Res* 1984; **44**: 3643–53.
23. Hsiang YH, Lihou MG, Liu LF. Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. *Cancer Res* 1989; **49**: 5077–82.
24. D'Arpa P, Berardmore C, Liu LF. Involvement of nucleic acid synthesis in cell killing mechanisms of topoisomerase poisons. *Cancer Res* 1990; **50**: 6919–24.
25. Heck MMS, Hittleman WN, Earnshaw WC. Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle. *Proc Natl Acad Sci USA* 1988; **85**: 1086–90.
26. Giaccone G, Capranico G, Beck H, Zunino F, Gazdar AF. Multidrug sensitivity phenotype of human lung cancer cells associated with topoisomerase II gene expression. *Cancer Res* 1992; **52**: 1666–74.

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