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Communication

Defective Brca2 influences topoisomerase I activity in mammalian cells★✪

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The Chinese hamster cell mutant V-C8 is defective in the *Brca2* gene (Kraakman-van der Zwet *et al.*, 2002, *Cell Biol.*; 22: 669). Here we report that V-C8 cells were 10-fold more sensitive to camptothecin, an inhibitor of topoisomerase I, than the parental V79 cells. The level of the relaxation activity of topoisomerase I in nuclear extracts was also lower (4-fold) in V-C8 than V79 cells, in spite of the fact that the level of the topoisomerase I protein was the same in these cells. The survival of V-C8 cells in the presence of camptothecin, the sensitivity of V-C8 topoisomerase I to camptothecin, and the level of the relaxation activity in V-C8 nuclear extract were almost completely restored by transfection of V-C8 cells with the murine *Brca2* gene or by the transfer of human chromosome 13 providing the *BRCA2* gene. These results indicate that the observed changes in the topoisomerase I activity in V-C8 are due to the defective function of the *Brca2* gene.

DNA double-strand breaks (DSBs) are generated following exposure of cells to ionizing radiation (IR) or to topoisomerase inhibitors, used as anticancer drugs. DSBs are also intro-

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Abbreviations: CPT, camptothecin; DSBs, DNA-double strand breaks; HR, homologous recombination.

duced during transposition events and meiotic recombination, and if not repaired, they can lead to genomic instability, genetic loss, or cell death (Frankenberg-Schwager & Frankenberg, 1990). In mammalian cells, DSBs are repaired *via* two repair mechanisms, either by non-homologous end joining (NHEJ) or by homologous recombination (HR) (reviewed in Karran, 2000).

DNA topoisomerases are nuclear enzymes responsible for the regulation of DNA topology. They are involved in basic DNA transactions during replication, transcription, and recombination (Ferguson & Baguley, 1994; D'Arpa & Liu, 1995; Gupta *et al.*, 1995; Watt & Hickson, 1994; Champoux, 2001; Wang, 2002). The intracellular level of topoisomerases is an important determinant of cellular sensitivity not only to topoisomerase inhibitors but also to ionizing radiation and oxidative damage (Davies *et al.*, 1988; 1990). DNA topoisomerases may participate in the repair of DSBs induced by both topoisomerase inhibitory drugs and radiation, in a common pathway (Pommier *et al.*, 1994; Weaver, 1996). Several authors have suggested that topoisomerase I could be involved in the repair of X-ray induced DNA damage (Kim *et al.*, 1992; Bootman *et al.*, 1989), and might have a function in UV excision repair as well (Thielman *et al.*, 1993). So far, a clear role for topoisomerase-dependent pathways in the cellular response to ionizing radiation has not been shown.

In order to study the involvement of topoisomerase I in the repair of ionizing radiation-induced DNA damage, an X-ray sensitive Chinese hamster cell mutant of complementation group XRCC11, V-C8 (Zdzienicka, 1995), was examined for its sensitivity to camptothecin, an inhibitor of DNA topoisomerase I. Recently, it has been shown that the V-C8 mutant is defective in the breast cancer susceptibility gene *Brca2*, and that this deficiency leads to hypersensitivity to various DNA-damaging agents with an extreme sensitivity toward interstrand DNA cross-linking agents (Kraakman-van der Zwet *et al.*, 2002). In addition, V-C8 cells show impaired repair of DSBs, radioresistant DNA synthesis (RDS) following ionizing irradiation, and chromosomal instability (Overkamp *et al.*, 1993; Verhaegh *et al.*, 1995). It has been recently shown that *BRCA2*-deficient human cells are deficient in the repair of DSBs and DNA cross-links through homologous recombination (Xia *et al.*, 2001).

Germline mutations in the *BRCA1* and *BRCA2* genes are associated with familial predisposition to breast and ovarian cancer (Rahman & Stratton, 1998). In addition, the status of both genes determines the phenotype of the tumor: BRCA1- or BRCA2-deficient tumors show marked sensitivity to ionizing radiation and to drugs that produce double-strand breaks (Xia & Powell, 2002). There are several reasons for the considerable interest in topoisomerase-targeting cancer treatment. First, topoisomerase poisons include some of the most active drugs that are currently available in chemotherapy. Active poisons of topoisomerase I include derivatives of camptothecin (CPT) (Slichenmayer *et al.*, 1993). Second, there is a potential in combining topoisomerase poisons with other types of therapy such as X-radiation (Boothman *et al.*, 1992).

In this report we demonstrate an increased sensitivity of V-C8 cells to CPT and a reduced topoisomerase I relaxation activity, as well as its increased sensitivity to CPT, measured in nuclear extracts from these cells. Both the CPT sensitivity of V-C8 cells and the *in vitro* activity of topoisomerase I are almost fully complemented by introduction of either human or murine *Brca2* gene.

MATERIALS AND METHODS

Cells and culture conditions. The V-C8 mutant cell line derived from the parental Chinese hamster V79 cells, and V-C8 cells with the human chromosome 13 providing the

BRCA2 gene, or with a bacterial artificial chromosome (BAC) containing murine *Brca2* have been described earlier (Overkamp *et al.*, 1993; Verhaegh *et al.*, 1995; Kraakman-van der Zwet *et al.*, 2002).

Cells were cultured as monolayers in plastic Petri dishes in Ham's F-10 medium (without hypoxanthine and thymidine), supplemented with 10% fetal calf serum (Gibco), penicillin (100 units/ml), and streptomycin (0.1 mg/ml). V-C8 mutant cells with the human chromosome 13 or with murine BRCA2 were cultured in the presence of 400 μg of geneticin (G418 sulfate)/ml. Cells were maintained at 37°C in a 5% CO_2 atmosphere humidified to 95–100%.

Chemicals. Camptothecin (CPT) was obtained from Sigma Chemical Co. Stock solution of CPT (10 mM): CPT was dissolved in dimethyl sulfoxide (Me_2SO) and kept at –20°C.

Survival curves. Cultures in exponential growth were trypsinized and 400–1000 cells were plated into 94-mm dishes in triplicate. Cells were exposed to CPT for 24 h. After the treatment medium was removed, the cells were rinsed twice with phosphate-buffered saline (PBS), then normal medium was added and cells were incubated for 8–10 days. After incubation, the dishes were rinsed with NaCl (0.9%), air dried, and stained with methylene blue (0.25%), and the visible colonies were counted. Each survival curve represents the mean of at least three independent experiments.

DNA topoisomerase I assay. Optimal conditions for the extraction of nuclei to examine DNA topoisomerase I activity from exponentially growing cells were achieved with the method described by Jonstone & Mc Nerney (1985) using 1 M NaCl. DNA content in the nuclei was determined by A_{260} measurements made in 1% SDS, 10 mM EDTA (pH 7.5).

The topoisomerase I assay measured the relaxation of supercoiled pBR322 plasmid DNA (100 ng/sample) by nuclear extracts according to Liu (1983). DNA electrophoresis was performed in 0.8% agarose; 2 mM EDTA, 40 mM Tris/acetate, pH 7.8, at 8 V/cm according to Sambrook *et al.* (1989). The results of the assays were quantified by densitometric scanning of negatives of photographed electrophoresis gels. One unit of activity relaxed 50% of the substrate DNA after 30 min at 37°C.

Immunoblot analysis. For immunoblot analysis whole cell extracts were made by resuspending cell pellets in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholic acid, 1 mM EDTA, pH 7.4, 1 mM dithiothreitol, 0.5 mg/ml Pefabloc, 1 μg/ml aprotinin, 1 μg/ml leupeptin), followed by sequentially snap-freezing on dry-ice and thawing at 30°C three times. In some experiments, a higher salt concentration (1 M NaCl) in the lysis buffer was used. Cell debris was removed by centrifugation at 14000 r.p.m. at 4°C for 15 min. Proteins (50 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane (Bio-Rad), and probed with polyclonal antiserum against human topoisomerase I (TopoGen Inc.) or monoclonal antiserum against actin (Santa Cruz). Membranes were then probed with horseradish-peroxidase-conjugated goat anti-rabbit or anti-mouse IgG, and antibody binding was detected by enhanced chemiluminescence (Amersham).

Protein determination. Protein concentration was determined by the method described by Bradford (1976) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Sensitivitity of V-C8 cells to camptothecin

Comparison of the clonogenic survival following treatment with camptothecin indicated that V-C8 cells were about 10-fold more sensitive to CPT than the parental V79 cell line (Fig. 1). Comparing the doses resulting in 10% survival assessed the degree of sensitiv-

ity. The hypersensitivity observed at the cellular level was accompanied by an increased sensitivity to CPT found for the relaxation activity measured in extracts from V-C8 nuclei (Fig. 2). At low concentration of the drug (5 μM CPT) the inhibition of the relaxation was about 50% for the V-C8 mutant and only about 10% for the control parental V79 cell line.

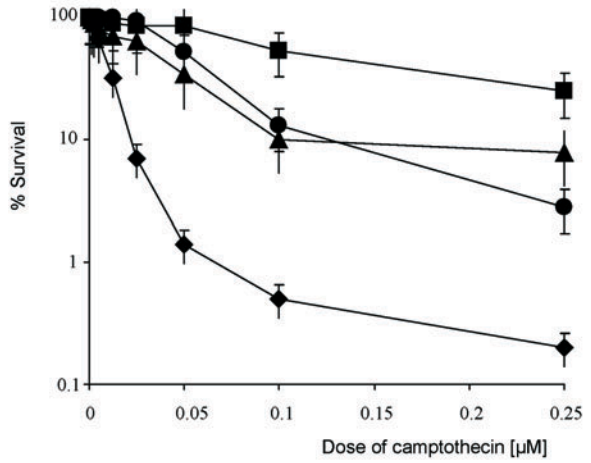

Figure 1. Cell survival after exposure to camptothecin.

The following cells were exposed to CPT: wild type V79 (●); V-C8 (◆); V-C8 cells with human chromosome 13 providing *BRCA2* (V-C8+#13) (▲), and V-C8 containing the murine *Brca2* gene (V-C8+*Brca2*) (■). Error bars represent the S.E.M.

The total relaxation activity in nuclear extracts from V-C8 cells was about 4-fold lower than in the extracts from V79 cells (Table 1). To examine whether the decreased activity was due to a lower level of the topoisomerase I protein, the expression of topoisomerase I was determined by immunoblot analysis for both cell lines. Proteins were extracted using either 0.15 M NaCl or 1 M NaCl. We checked that extractability of topoisomerase I was the same in both cases (not shown). Results presented in Fig. 3 show that the levels of topoisomerase I protein were similar in V-C8 and V79 cells, indicating that the lower relaxation activity in nuclear extracts from V-C8 cells did not result from a diminished expression of topoisomerase I. This suggests that Brca2-deficiency affects the specific activity of topoisomerase I.

Complementation of CPT sensitivity by the *Brca2* gene

To examine whether the reduced topoisomerase activity in V-C8 cells was really due to the defective Brca2, the same experiments were performed in complemented V-C8 cells. As shown in Fig. 1, the hypersensitivity of V-C8 cells to CPT was complemented in V-C8 cells containing the murine *Brca2* gene or human chromosome 13 providing the human *BRCA2* gene. Also, the hypersensitivity to CPT of the relaxation activity was complemented by human or murine *Brca2* (Fig. 2).

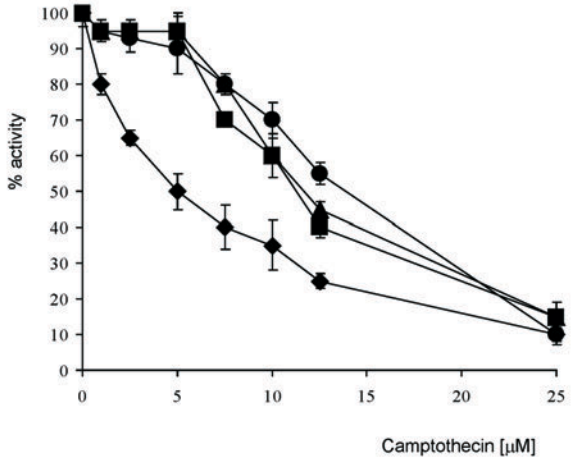

Figure 2. Inhibitory effect of camptothecin on topoisomerase I activity.

The relaxation activity of topoisomerase I was measured in nuclear extracts from V79 (●); V-C8 (◆); V-C8 cells with human chromosome 13 providing *BRCA2* gene (V-C8+#13) (▲), and V-C8 containing the murine *Brca2* gene (V-C8+*Brca2*) (■) in the presence of CPT. Presented data are mean values of at least three independent experiments. Error bars represent the S.E.M.

However, levels of topoisomerase I protein in nuclear extracts from V-C8+#13 and V-C8+*Brca2* cells were similar to those of V79 and V-C8 cells (Fig. 3). Taken together, these results show that the Brca2-deficiency in V-C8 cells is directly associated with the increased sensitivity of this mutant to CPT. In addition,

the presented results point to topoisomerase I as a factor responsible for the hypersensitivity to CPT of V-C8 cells, although the mechanism of this phenomenon has not been revealed in this work. Restoration of topoisomerase activity in V-C8 cells by complementation with human or murine *Brca2* suggests an interaction between topoisomerase I and Brca2. As mentioned previously, topoisomerases are involved in the regulation of DNA topology in several processes, including DNA replication (Champoux, 2001; Wang, 2002). BRCA2 has been found to play an important role in the repair of DSBs through HR. During S-phase BRCA2 interacts with several proteins directly involved in HR, including RAD51 and BRCA1 (for a review see: Bernstein *et al.*, 2002). Therefore, our data might indicate that topoisomerase I is tightly bound to the HR-machinery as well. Recent studies suggest that topoisomerase I could also potentially interact with at least three other proteins involved in the repair of DNA damage, including the BRCA1-associated ring protein BAR1 (Trzcińska *et al.*, 2002).

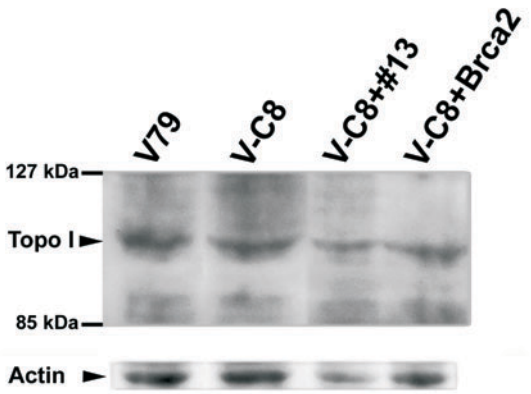

Figure 3. Immunoblot analysis of the hamster topoisomerase I protein.

Topoisomerase I protein was analyzed in V79, V-C8, V-C8+#13 and V-C8+*Brca2* cells with polyclonal antiserum against human topoisomerase I. Equal loading was confirmed by re-probing of the blots with monoclonal antiserum against actin. The ratio of actin to topoisomerase I levels in each sample, shown by densitometric analysis, is 1 : 1 (± 5%).

We can exclude at least some effects which could be responsible for the observed differences in topoisomerase I activity in BRCA2-deficient cells connected with p53 functional status, due to the presence of mutated and nonfunctional p53 protein in V79 cells (Chaung *et al.*, 1997). Our results indicate that the observed changes in the relaxation activity of topoisomerase I in V-C8 cells, as well as its sensitivity to CPT do not result from an altered expression of the topoisomerase I protein. Thus, either modification of the topoisomerase I protein or a change in its interaction with other proteins should be considered as the reason of the altered activity of topoisomerase I in V-C8 cells. Topoisomerase I undergoes several post-translational modifications, e.g. phosphorylation (Pommier *et al.*, 1990) and poly(ADP)-ribosylation (Ferro *et al.*, 1983). Each of them might affect both the relaxation activity and the sensitivity of the enzyme to CPT. Further studies are required to understand the mechanism responsible for the changes in the topoisomerase I activity, which might be useful in cancer therapy.

Table 1. Activity of topoisomerase I.

Nuclear extracts of the parental cell line V79, mutant V-C8, as well as mutant V-C8 complemented by human or murine *Brca2* gene were used. The topoisomerase I assay measured the relaxation of pBR322 plasmid DNA.

Cell line	Activity of topoisomerase I (U/μg DNA)*
V79	549
V-C8	121
V-C8 + human chromosome 13 (V-C8+#13)	503
V-C8 + murine *Brca2* (V-C8+*Brca2*)	498

*One unit relaxed 50% of substrate DNA after 30 min at 37°C.

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