RAD51 OVEREXPRESSION AND RESISTANCE TO GENOTOXIC AGENTS. A STUDY IN THE FISSION YEAST *SCHIZOSACCHAROMYCES POMBE*

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RAD51 OVEREXPRESSION AND RESISTANCE TO GENOTOXIC AGENTS. A STUDY IN THE FISSION YEAST SCHIZOSACCHAROMYCES POMBE **(Abstract): Background:** Many cancer cell lines have been found to overexpress the recombinase Rad51. The overexpression is associated with increased invasive potential and resistance to DNA-damaging therapeutic agents. This has been attributed to an increased capacity of cells over-expressing Rad51 to repair DNA lesions or to a genetic stabilization of the genome. **Aim:** As the explanations are somewhat controversial, we attempted to reproduce overexpression in the unicellular eukaryote *Schizosaccharomyces pombe* to have a simpler tool to study the problem of Rad51 overexpression and its induced resistance to DNA-damaging agents. **Methods:** We used the *nmt1* promoter inserted upstream of *rad51*gene to induce its overexpression and studied the phenotype of the transformed strain, especially its sensitivity to camptothecin and hydroxyurea. **Results:** We found that overexpression induced sensitivity to the two drugs even when it was associated with the deletion of a recombination mediator *rad22/rad52* gene. However, when overexpression was associated with the deletion of the helicase-encoding *fbh1* gene, the sensitivity to camptothecin was diminished. **Keywords**: RAD51 OVEREXPRESSION, HOMOLOGOUS RECOMBINATION, RAD22, FBH1.

Rad51 protein is a central player involved in repair of the DNA double-strand breaks by homologous recombination. Rad51 is a component of the recombination nucleo-protein filament: a DNA single stranded overhang (resulting from the end processing of a double-stranded end) wrapped around a Rad51 polymer in a helical manner. The nucleofilament invades the double helix of a sister chromatid or a homologous chromosome forming the so called D loop, a structure that initiates homologous recombination (1). Rad51 is essential in mammalian cells; mouse embryos carrying deletion of the *rad51* gene are unviable (2).

Publications issued after 2002 have reported a Rad51 overexpression in many (over 50%) of human cancers (3). The overexpression exceeds about 2-7 fold the level of the expression in normal parental cells, in terms of the Rad51 protein amount. It is due to an excessive transcription of the gene (4). Some activating influence over the genes promoter occurs. In a series of 13 cancer cell lines, the activity of the *rad51* promoter was tested with a different gene reporter (luciferase) and a dramatically increased level of the reporter protein occurred (several hundred fold) in cancer cells when compared to normal fibroblasts (5). In the same cell lines the

Rad51 mRNA and protein were increased only about six fold, which means that a limiting action on the transcript's level also occurs. The overexpression confers either increased invasive potential and/or increased resistance to genotoxic agents used for cancer therapy (4,6).

Overexpression has *causes* and *consequences*. Both causes and consequences are important to be known for cancer prevention and therapy. A way of discovering them is to look for genome modifications accompanying the Rad51 overexpression. Some of these are the ones leading to losses of function of p53, BRCA1, BRCA2 and gain of function of the oncoprotein BCR/ABL (3).

p53 is a well known tumor suppressor. It is a transcription factor important for the regulation of cell cycle. p53 is a negative regulator of the *rad51*gene expression by directly inhibiting its transcription (6). BCR/ABL is an activator of the *rad51* expression through STAT5-dependent transcription of *rad51* and inhibition of Rad51 protein cleavage by caspase-3 (7). BRCA1 and 2 are proteins involved in homologous recombination like Rad51 itself (8). Their loss-of-function mutant alleles may be involved in Rad51 overexpression through their deficiency in repairing DNA lesions, which is deleterious for the cell viability and favor selection of cells overexpressing Rad51 to compensate for their own defect. In this one vision, the BCR/ABS gain-of-function and p53, BRCA1 and BRCA2 lss-of-function are causes of Rad51 overexpression. However, a reverse scenario is also possible: Overexpression of Rad51 could be the cause of genetic instability (also deleterious for the cell growth) and then selection of mutations to compensate for it in the genes coding for either p53 or BRCA1 or BRCA2 would occur as a consequence (9). The main support of this hypothesis is that Rad51 overexpression is deleterious in normal cells but it is beneficial for cells with BRCA1 or BRCA2 loss-of-function (10).

The resistance of cancer cells overexpressing Rad51 to DNA damaging agents (MMS, camptothecin, cisplatin) is of utmost interest for cancer chemotherapy (11), but it is questionable whether this is due to overexpression itself or to the accompanying mutations.

We assumed that the use of simple eukaryotic organisms such as yeasts could shed some light on the problem of Rad51 overexpression. It would permit a better differentiation between causes and consequences of this Rad51 status. Rad51 can be overexpressed by putting its gene under the control of an artificial active promoter and testing of the genomic modified strain for resistance/sensitivity to DNA-damaging agents can be performed. In this way, the overexpression being unaccompanied by other mutations, its sole influence on the resistance/sensitivity can be assessed. We carried out the genomic modification and studied its effects in the yeast *Schizosaccharomyces pombe*.

A previous study (12) has shown that overexpression of Rad51/Rhp51 in *Schizosaccharomyces pombe* had deleterious effects on the strain which carried it. Cells had reduced viability and a defect in chromosome segregation at mitosis due to disruption of microtubule attachment to the chromosomes. In this study we confirm loose of viability (to a lesser degree than reported previously (12) and we also report an increased sensitivity to camptothecin and hydroxyurea of transformed cells. However, in strains lacking the *fbh1* gene

(coding for a helicase/translocase which opposes the nucleofilament formation) (13) overexpression of Rad51 conferred lesser sensitivity compared to the strains with normal activity of Fbh1.

MATERIAL AND METHODS

The *S. pombe* strains used in this study, their complete genotype, and their source are presented in tab 1.

Strains AMC 504, AMC60 and 272 were kind gifts from A.M. Carr, University of Sussex UK. The other strains were constructed by us as follows: The *S. pombe* active promoter *nmt1* was inserted upstream of rad51 as described (14, 15). The strain carrying *E-rad22* was obtained similarly but accidental mispriming resulted in a hypomorphic allele (with partial loss-of-function) of rad22. The other strains were derived by standard crosses of 251 (nmt1-rad51) with 272 (fbh1-d) and 266 (E-rad22), followed by sporulation and dissection of the tetrads to obtain spore-clones. They were tested phenotypically (resistance to geneticin meant deletion of fbh1) and genotypically (PCR amplification with a pair of primers, one of them being identical with a sequence in *nmt1* meant overexpression of *rad51*).

TABLE I

***Schizosaccharomyces pombe* strains used in this study**

Strain Nr (in our collection)	Relevant genotype and current name	Complete genotype	Source
AMC 504	rad51+ rad22+ fbh1+ (WT)	*ade6-704 ura4-d18 leu1-32 h-*	A.M. Carr
272	fbh1-d	*fbh1::kanr ade6-704 ura4-d18 leu1-3 2 h+*	A.M.Carr
AMC60	rad51-d	*rad51::kanr ade6-704 ura4-d18 leu1-32 h-*	A.M.Carr
251	nmt1-rad51	*nmt1-rad51 ade6-704 ura4-d18 leu1-32 h-*	This work
339	nmt-rad51 fbh1-d	*nmt1-rad51 fbh1::kanr ade6-704 ura4-d18 leu1-32*	This work
266	E-rad22	*E-rad22 ade6-704 ura4-d18 leu1-32 h-*	This work
317	E-rad22	*E-rad22 ade6-704 ura4-d18 leu1-32 h+*	This work
318	E-rad22 nmt1-rad51	*E-rad22 nmt1-rad51 ade6-704 ura4-d18 leu1-32 h+*	This work
319	E-rad22 nmt1-rad51	*E-rad22 nmt1-rad51 ade6-704 ura4-d18 leu1-32 h-*	This work

Culture medium and chemicals: The standard yeast extract agar (YEA) medium was used in all tests (yeast extract 0.5%, glucose 1%) to which camptothecin (Cpt) (Sigma-Aldrich USA) or hydroxyurea (HU, Sigma-Aldrich USA) were added where indicated and in the concentrations shown in figs 1 and 2. The kanamycin-like antibiotic G418 (geneticin Sigma-Aldrich USA) was added at the concentration of 400 mg/l to identify *kanr* (gene which replaces *fbh1* or *rad51*).

Testing of genotoxin sensitivity/resistance: The semi-quantitative "spot test"

was used (13).

RESULTS

Overexpression of *rad51* sensitizes cells to hydroxyurea and camptothecin. Insertion of a variant of the *nmt1* promoter is a frequently used method for overexpressing genes in *Schizosaccharomyces pombe* (14, 16).We first tested the strain with *rad51* placed under the control of the hyperactive promoter *nmt1* for its phenotype. The viability of untreated cells, estimated by the percentage of dead (magdala red dye accumulating) cells in YEA was slightly lower than of the wild-type (WT) cells (not shown).This was not so significant as to be apparent on spot tests (fig. 1, no genotoxin) as reported previously (12). This is probably due to the fact that in our strain there was a single gene under the *nmt1* control (as *nmt1* was inserted upstream of the single chromosomal gene) while Kim et al (12) constructed overexpressing strains using many copies of a plasmid carrying *rad51/rhp51* under the control of *nmt1* promoter. The sensitivity of our strain overexpressing *rad51* to hydroxyurea and camptothecin was higher compared to WT and even higher than sensitivity of the strain deleted of *rad51* (fig. 1 A).

This is not different from other types of wild-type cells, including human cells and even some cancer cells (3) which are also more sensitive when Rad51 is overexpressed. Some types of cancer cells however, especially the ones carrying mutations in BRCA2 (10) are less sensitive to DNA-damaging chemotherapeutics when Rad51 is overexpressed. We tried to reproduce the situation in *S. pombe* by combining the partial loss-of-function of Rad22 (the functional equivalent of BRCA2) (17) with Rad51 overexpression.

The double mutant E-rad22 nmt1-rad51 was not less sensitive than the single-mutant E-rad22. Rad51overexpression did not compensate for the Rad22 deficiency; in that regard the situation described in cancer cells could not be reproduced in *Schizosaccharomyces pombe*. (fig. 1B)

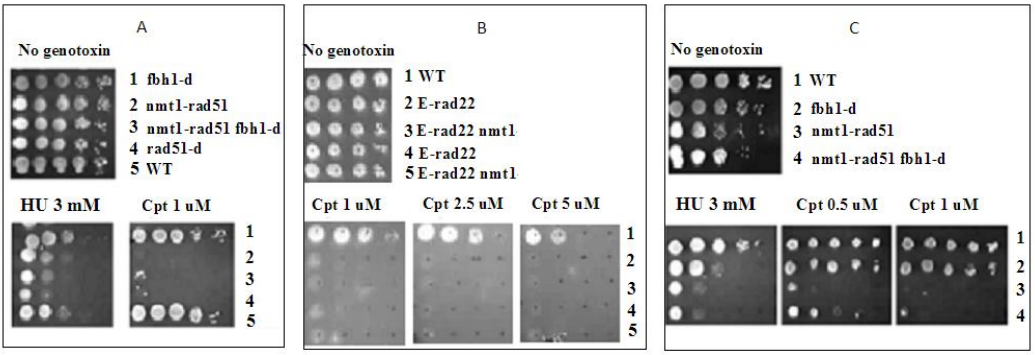

Fig. 1, A, B, C. Viability of *S. pombe* strains with and without mutations at *rad51*, *rad22*, or *fbh1* loci.

Cells were grown to stationary phase and then serially diluted to have 10^5, 10^4, 10^3, 10^2 or 10 cells/5 µl and then 5 µl of each dilution, in the decreasing order from

left to right, was dropped in a row on plates containing the solid medium YEA with or without the genotoxins in concentrations as mentioned . The plates were photographed after 5 days of incubation at 30^{o}C. Panel A: Strains overexpressing Rad51 are hypersensitive to hydroxyurea and camptothecin. Panel B: The Rad22/Rad52 loss-of-function cannot be compensated by overexpression of Rad51. The strains 2 and 4 (317 and 266) were identically mutated at rad22 but they were derived independently and had slightly different genetic backgrounds. The same observation is available for strains 3 and 5 (double mutant's numbers 318 and 319). (tab. I) Panel C: Deletion of the gene *fbh1* alleviates the hypersensitivity to camptothecin of the strain overexpressing Rad51.

Deletion of *Fbh1* gene alleviates the hypersensitivity to camptothecin of strains overexpressing Rad51.

One question that could be raised in respect to the phenotype of Rad51 overexpression is whether it is due to the well known function of the protein in forming the nucleofilament necessary for recombination repair (1) or to a different unknown function. To answer this question we combined overexpression of Rad51 with the lack of a helicase/traslocase function that opposes the nucleofilament formation. Of the several helicases involved we chose Fbh1 which has a homologue in human cells (18, 19). We thus combined *nmt1-rad51*construct with the deletion of *fbh1* gene in the same strain.

We expected that if the excessive formation of nucleofilament had been responsible for genotoxin sensitivity it would have worsened the effect of overexpression (for example to result in less viability even in the cells untreated with genotoxins). This expectance was not confirmed. There was little difference (if any) between the viability of single mutant nmt1-rad51 and double mutant nmt1-rad51 fbh1-d in the media without genotoxin or with hydroxyurea. However, in the media containing camptothecin a clear loss of sensitivity could be perceived in the case of the double mutant nmt-rad51 fbh1-d. In other words, some resistance to camptothecin was visible on the background of Fbh1 loss of function. (fig. 1C)

DISCUSSION

We showed that overexpression of Rad51 sensitized *S. pombe* cells to the action of DNA- damaging drugs hydroxyurea and camptothecin. These two drugs damage DNA by different mechanisms but eventually result in double-strand breaks which are lethal if unrepaired. Their repairing depends on Rad51. Therefore, the absence of Rad51 (as in the case of the strain with the deletion of the gene rad51 (fig.1 A) sensitizes cells to HU and Cpt. We showed in this study that also an excess of Rad51 sensitizes cells to hydroxyurea and camptothecin (Panels A and C).

Is the deleterious effect of Rad51 overexpression due to an excessive formation of nucleofilaments ? The double-strand repair by recombination needs Rad51-dependent nucleofilament assembly in one step of the process and its helicase-dependent disassemble in another step. An excess of Rad51 may distort this balance and lead to an inefficient repair. In this case, the lack of helicase function will augment the unbalance and make the process even less efficient. Therefore we eliminated one of helicase functions by deleting the gene *fbh1* encoding the helicase Fbh1 and found that

the deletion did not add up at the sensitivity to genotoxins or worsen the viability of the cells carrying the double mutation *nmt1-rad51 fbh1-d* (figs. 1 A and C), as expected, were the recombination defects the main cause of the sensitivity. We presume that other functions of Rad51 were in cause. A previous report has shown that Rad51 is involved in cell cycling and mitosis (12), possibly by a different mechanism than that of nucleofilament formation.

Deletion of the helicase/translocase-encoded *fbh1* on the background of Rad51 overexpression had an unexpected effect: the diminution of sensitivity to camptothecin. (fig. 1 C). This is a reminiscent of the resistance of cancer cells overexpressing Rad51 to DNA-damaging chemotherapy. In our view, this finding may be interpreted in two alternative ways: 1. Hypersensitivity to camptothecin of the strain overexpressing Rad51 is not due to its effect over the nucleofilament formation and efficiency of recombination but to a different mechanism. Recombination is not affected as long as Fbh1 helicase/ translocase exists. When the helicase function does not exist, repair by recombination is more efficient and this leads to a relative resistance to camptothecin - relative because the main mechanism which is the cause of sensitivity is not suppressed - (the physiologically-based hypothesis). 2. The double genomic modification which confers the Rad51 overexpression on the background of the Fbh1 absence leads to a genomic instability and to a new mutation conferring resistance to camptothecin. The resistant cells select in the medium containing camptothecin (the genomic instability hypothesis). We cannot as yet distinguish between these two hypotheses, but the second one (genetic instability hypothesis) can be checked by genetic methods: selection of the clone presenting stably a less sensitivity to camptothecin, loss and regain of this capacity in crosses and, eventually, identification the mutated gene. That we are going to carry out further.

Relevance of our findings to the problem of cancer cells overexpressing Rad51 and their resistance to chemotherapeutics: We started this study as an attempt to reproduce in fission yeast and explain the resistance to DNA-damaging agents of cells overexpressing Rad51. Our attempt was justified by the fact that the action of DNA-damaging chemotherapeutics, the mechanisms of DNA repair and the proteins involved are similar in human cells and in yeasts. We did not find that fission yeast overexpressing Rad51 was resistant to any of two DNA-damaging agents hydroxyurea and camptothecin either Rad51 overexpression singly or accompanied by the functional equivalent of BRCA2 (Rad52/Rad22) loss-of-function. This is different from cancer cells in which association between Rad51 overexpression and BRCA2 loss-of-function is thought to cause resistance to DNA-damaging agents. However, one of our findings in this work on *S. pombe* points to recombination being involved in resistance. This is the alleviation of sensitivity to camptothecin of fission yeast cells deleted for the gene coding for helicase/translocase Fbh1. Fbh1 protein is also present in mammalian cells (19).

CONCLUSIONS

This work attempted to reproduce in fission yeast the Rad51 overexpression and resistance to DNA-damaging chemotherapeutics described in cancer cells. We found that overexpression of Rad51 in fission yeast sensitized cells to DNA damaging

agents, but sensitization was alleviated by the loss-of-function of the helicase/translocase Fbh1. This draws attention to a protein also present in human (and therefore in cancer) cells that may be involved in resistance either by its role in recombination or as a genetic stabilizer.

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NEWS

MULTIFOCAL GENITAL HERPES SIMPLEX VIRUS TYPE 2 INFECTION

Genital HSV reactivation is thought to be anatomically and temporally localized, coincident with limited ganglionic infection. Subclinical shedding episodes are the most common form of HSV-2 reactivation, with host clearance mechanisms leading to rapid containment.To precisely define patterns of anatomic reactivation, Johnston and co. divided the genital tract into a 22-region grid and obtained daily swabs for 20 days from each region in 28 immunocompetent, HSV-2 seropositive persons. HSV was detected via PCR and sites of asymptomatic HSV shedding were biopsied within 24 hours. They quantified CD4+ and CD8+ T cells by immunofluorescence, and HSV specific CD4+ T cells were identified by intracellular cytokine cytometry. HSV was detected in 868 of 11,603 genital swabs at a median of 12 sites per person. Bilateral HSV detection occurred on 83 days with shedding, and the median quantity of virus detected/day was associated with the number of sites positive ($p<0.001$). In biopsies of asymptomatic shedding sites, we found increased numbers of CD8+ T cells compared to control tissue (27 vs. 13 cells/mm^2, $p=0.03$) and identified HSV specific CD4+ T cells. HSV reactivations emanate from widely separated anatomic regions of the genital tract and are associated with a localized cellular infiltrate that was demonstrated to be HSV-specific in 3 cases. The authors concluded that HSV-2 reactivation can be detected at multiple, bilateral sites in the genital tract, suggesting that HSV establishes latency throughout the sacral ganglia. In addition, genital biopsies from sites of asymptomatic HSV shedding have increased numbers of CD8+ T cells compared to control tissue, and HSV-specific CD4+ T cells are found at sites of asymptomatic shedding. These findings suggest that that widespread asymptomatic genital HSV-2 shedding is associated with a targeted host immune response and contributes to chronic inflammation throughout the genital tract. (Johnston C, Zhu J, Jing L, Laing KJ et al. Virologic and immunologic evidence of multifocal genital herpes simplex virus type 2 infection. *J Virol*. 2014 Feb 19. [Epub ahead of print] PMID: 24554666)

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