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Involvement of Brca1 in DNA Interstrand Cross-link Repair Through Homologous Recombination-independent Process

Jeanho Yun*

Medical Research Center for Cancer Molecular Therapy, College of Medicine, Dong-A University, Busan 602-714, South Korea, Department of Biochemistry, College of Medicine, Dong-A University, Busan 602-714, South Korea

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Hypersensitivity of cells lacking Brca1 to DNA interstrand cross-link (ICL) agents such as cisplatin and mitomycin C (MMC) implicates the important role of Brca1 in cellular response following ICL treatment. Brca1 plays an essential role in DNA double-strand break (DSB) repair through homologous recombination (HR) -dependent and - independent process. Recently, our group has been reported that Brca1 involves in cellular ICL response through HR-dependent repair process (Yun J. et al., Oncogene 2005). In this report, the involvement of Brca1 protein in HR-independent repair process is examined using isogenic $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ mouse embryonic fibroblast (MEF) and psoralen cross-linked reporter reactivation assay. Brca1-deficient MEFs showed significantly low HR-independent repair activity compare to Brca1-proficient MEFs. Hypersensitivity to MMC and ICL reporter repair activity were restored by the reconstitution of Brca1 expression. Interestingly, MEFs expressing exon 11-deleted isoform of Brca1 (Brca1 $^{\Delta 11/\Delta 11}$) showed high resistance to MMC and ICL reporter repair activity comparable to Brca1-reconstituted MEFs. Taken together, these results suggest that Brca1 involves in ICL repair through not only HR-dependent process but also HR-independent process using N-terminal RING finger domain or C-terminal BRCT domain rather than exon 11 region which mediate interaction with Rad50.

Key words – Brca1, mitomycin C, DNA interstrand cross-link (ICL) repair, Recombinant-independent

DNA interstrand cross-links (ICLs) are among the most toxic of all DNA lesions. Since ICL prevents DNA strand separation and therefore can block essential cellular process like DNA replication, transcription and recombination so that ICLs finally induce cell cycle arrest, chromosomal rearrangement and cell death. ICL agents such as cisplatin, mitomycin C (MMC) and nitrogen mustard are widely used for potent anticancer treatment[1], but the molecular detail of ICL repair is still not well understood in higher mammalian cells.

Although the detailed sequence of events remains to be determined, previous studies suggests that nucleotide excision repair (NER) and homologous recombination (HR) are involved in ICL repair (reviewed in[2]). It is likely that conservative recombination using undamaged homologous regions as a donor may be a main repair pathway for an error-free manner[3]. However, in *Escherichia coli* and yeast, recombination-independent mechanisms of ICL repair have been identified in addition to HR-dependent repair pathways [4,5]. Moreover, recombination-independent pathway in mammalian cells was identified recently as well[6].

*Corresponding author
Tel : +82-51-240-2919, Fax : +82-51-241-6940
E-mail : yunj@dau.ac.kr

The importance of understanding ICL repair is highlighted by the hypersensitivity to ICL agents in cells lacking breast cancer tumor suppressor genes *Brca1* and *Brca2*[7-9]. Substantial evidence exists to support a role of Brca1 in mediating the cellular response to DNA damage, through HR, nonhomologous end joining (NHEJ) and NER process (reviewed in[10,11]). Although the hypersensitivity of Brca1 deficient cell to ICL agents has been observed in previous studies, the detail mechanism of sensitivity still remains to be studied. Previously, our research group reported the role of Brca1 in recombination-dependent repair process of DNA ICLs[12]. But, the role of Brca1 in recombination-independent repair process was never been studied.

Brca1 gene contains 24 exons and encodes a large protein of 1863 amino acid. At its amino terminus, Brca1 harbors a structurally conserved RING finger domain and the C-terminal region contains two tandem BRCT (Brca1 C-terminal) domains. The central region, which constitutes about 60% of the protein, is encoded solely by exon 11. A host of proteins, including DNA repair proteins, Rad50, Rad51, associate with this part of Brca1[10,11] suggesting the exon 11 region harbors an important function in DNA damage response.

In the present study, I examined the role of Brca1 in re-

combination-independent DNA ICL repair using isogenic $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ MEFs. In the psoralen ICL repair assay, Brca1-deficient MEFs found to be highly defective in the recombination-independent repair of ICLs compared to Brca1-proficient MEFs. The reconstitution of Brca1 expression in Brca1-deficient MEFs restored repair activity and resistance to ICL agent MMC suggesting that Brca1 plays an important role in recombination-independent ICL repair process. Importantly, Brca1 exon11-deleted MEFs ($Brca1^{\Delta 11/\Delta 11}$) showed comparable resistance against MMC and ICL repair activity to Brca1-reconstituted MEFs suggesting N-terminal RING domain and C-teminal BRCT domain of Brca1 protein are important for ICL repair function.

Materials and Methods

Cell lines

$p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ MEFs were generated previously [13]. MEF expressing exon 11-deleted isoform of Brca1 ($Brca1^{\Delta 11/\Delta 11}$) was a gift from Dr. X. Deng[14]. All MEF cell lines used in this report cultured in DMEM high glucose media containing 5% of FBS.

Transfection and reporter reactivation assay

Psoralen cross-linked or unmodified control luciferase reporter substrate were generously provided by Dr. L. Li [6]. For the luciferase reporter reactivation assay, 0.5 to 2 ng of posralen cross-linked or unmodified control reporter substrate was transfected in 35 mm plates using FuGene-6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's recommendations. Cells were harvested 40 hrs after transfection, and the luciferase activity was assayed using the Luciferase Assay System (Promega) and luminometer (LB953, Berthold). To examine Brca1 effect, 0.5 μg of mouse Brca1 expression plasmid was cotransfected with luciferase reporter plasmid. The relative repair efficiency was calculated as the percentage of luciferase activity from cross-linked reporter to that of the unmodified reporter [repair efficiency (%) = (luciferase activity of ICL plasmid/ luciferase activity of control plasmid)×100]. Each transfection experiments were performed in duplicate or triplicate and each data were the results of at least three independent transfections.

Generation of Brca1 Reconstitution cell line and Brca1 protein detection

A retroviral vector in which murine Brca1 expression is directed by the viral promotor in the long terminal repeat generously provided by Dr.Olga Aprelikova (NCI, NIH, Bethesda, MD)[15]. This plasmid was transfected into Phoenix ampho cell lines, which enable a rapid production of high titer, helper-free retroviruses using transient transfection [16]. Infectious retrovirus was produced within 48 hrs following transfection of Brca1 construct. The retroviral supernatant was harvested at 48 hrs post-transfection and stored at -80℃.

A 1ml portion of fresh thawed retroviral supernatant with polybrene at a final concentration of 2 μg/ml was used for infection of $p53^{-/-}Brca1^{-/-}$ MEFs. The infected cells were selected against 4 μg/ml puromycin, 10-14 days later, the resistant clones were picked up and tested for Brca1 expression. The Brca1 protein expression was examined using anti-murine Brca1 polyclonal antibody with immunoprecipation-western blot analysis. For generation of anti-murine Brca1 antibody, amino acids 788 to 1135 of mouse Brca1 were fused to glutathione S-transferase (GST) and the fusion protein was used as an antigen for generating mouse polyclonal antisera.

MMC clonogenic survival assay

For MMC survival assay, 1×10^3 or 4×10^3 cells were seeded in 100mm plate and exposed to various MMC doses for 1 hr and then rinsed three times with PBS. 10-12 days later, the colonies were fixed and stained with methylene blue, and counted. The number of colonies obtained with untreated cells was corrected for plating efficiency and normalized to 100% survival. Survival experiments were performed in duplicate or triplicate.

Results and Discussion

$p53^{-/-}Brca1^{-/-}$ MEFs are defective in recombination-independent ICL repair

In order to examine ICL repair activity in $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ MEFs, we employed an ICL reporter reactivation assay[6]. To measure ICL repair activity, a single psoralen ICL was introduced into a luciferase reporter plasmid between the cytomegalovirus promoter and luciferase coding sequence. The unmodified control reporter plasmid and cross-linked reporter plasmid were identical except that a single defined ICL was present in the latter. Thus, the expression of luciferase from cross-linked reporter plasmid became dependent upon removal of the ICL. Quantification

of the ICL repair efficiency can be achieved by normalizing the luciferase activity from cells transfected with cross-linked plasmid against that of cells transfected with an unmodified reporter plasmid.

When the same amount of both plasmids was transfected in parallel to $p53^{-/-}$ MEFs, we observed about 30% of reactivation efficiency (Fig. 1). However, $p53^{-/-}Brca1^{-/-}$ MEFs showed significantly lower repair efficiency compare to $p53^{-/-}$ MEFs. This low repair efficiency was seen at all over substrate DNA amount range. This result provides clear evidence that Brca1 participate in ICL repair through recombination-independent pathway.

ICL repair activity is restored by reconstitution of Brca1 expression

To further evaluate the role of Brca1 in recombinant-independent ICL repair, I first cotransfected wild-type mouse Brca1 expression plasmid with psoralen cross-linked or unmodified control reporter plasmid. As shown in Fig. 2, cotransfection of Brca1 expression plasmid led complete restoration of ICL repair activity suggesting Brca1 function is required for the efficient repair of ICL.

We also reintroduced the Brca1 protein expression into Brca1-deficient MEFs using a retrovirus encoding murine Brca1 as described in Materials and Methods. After infection

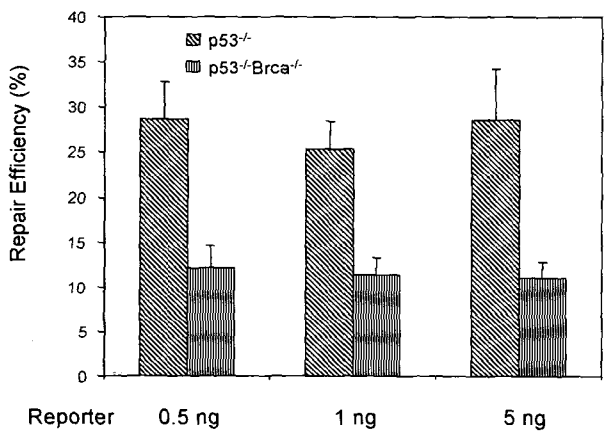

Fig. 1. $p53^{-/-}Brca1^{-/-}$ MEFs are defective in recombination-independent ICL repair. 0.5 to 5 ng of psoralen cross-linked or unmodified control luciferase reporter plasmid was transfected into $p53^{-/-}$ or $p53^{-/-}Brca1^{-/-}$ MEFs. Luciferase activities were measured 40 hrs after transfection and the relative repair efficiency were calculated as the percentage of luciferase activity from cross-linked reporter to that of the unmodified reporter. All results are means of at least three independent experiments and error bars show the standard deviation.

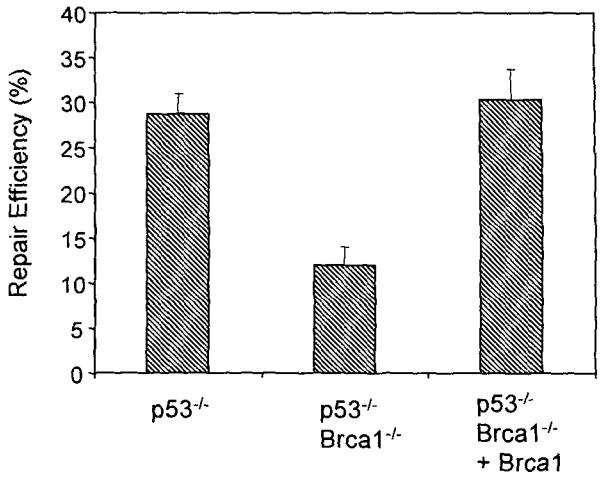

Fig. 2. Recombination-independent ICL repair activity restored by ectopic expression of Brca1. 0.5 μg of mouse wild type Brca1 expression plasmid was cotransfected with 0.5 ng of psoralen cross-linked or unmodified control luciferase reporter plasmid. All results are means of at least three independent experiments and error bars show the standard deviation.

with Brca1-retrovirus, cells were selected with puromycin and the individual colonies were isolated. Brca1 expression of clones was examined and western blot analysis showed that the Brca1-reconstituted clone number 3 (R#3) expressed the comparable level of Brca1 protein to $p53^{-/-}$ MEFs (Fig. 3a). To test whether reconstitution of Brca1 expression restores the ICL repair activity, Brca1 R#3 together with $p53^{-/-}$ and $p53^{-/-}$ $Brca1^{-/-}$ MEFs were subjected to ICL reporter assay. The Brca1-reconstituted MEFs showed significant increase of repair efficiency, albeit not to the same level as $p53^{-/-}$ MEFs (Fig. 3b). These results confirm that the defect in recombination-independent ICL repair of $p53^{-/-}$ $Brca1^{-/-}$ MEFs is due to the absence of Brca1 function.

Brca1-exon11 deletion MEFs exhibit high ICL repair activity

Previous studies demonstrated that exon 11 region of Brca1 is important for DNA damage response. Brca1-exon 11 deletion MEF ($Brca1^{\Delta 11/\Delta 11}$) showed various defect in DNA damage response such as abnormal formation of Rad51 foci, hypersensitivity to γ[gamma]-irradiation, defective G2-M checkpoint and improper centrosome amplification [14,17,18]. Rad50 and Rad51 interact with Brca1 through exon 11 region indicating this region is crucial for DNA repair function of Brca1. Thus, it is expected that $Brca1^{\Delta 11/\Delta 11}$ MEFs shows defect in ICL repair too.

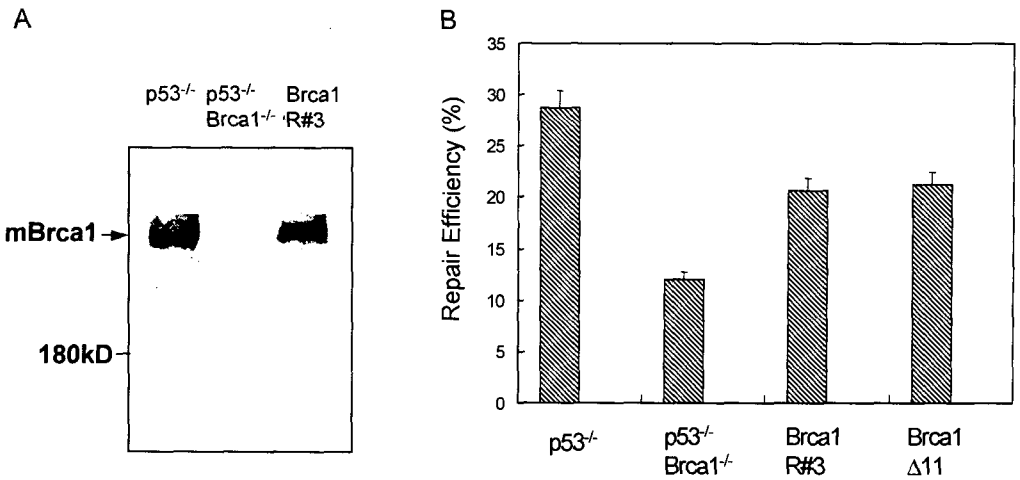

Fig. 3. ICL repair acitivity restored by reconstitution of wild type Brca1 gene. (A) Brca1 protein expression in $p53^{-/-}$, $p53^{-/-}$ $Brca1^{-/-}$ and Brca1-reconstituted clone #3 (R#3) was examined with immunoprecipitation-Western blot analysis using anti-murine Brca1 antibody. (B) Repair efficiency of Brca1-reconstituted clone (R#3) and Brca1-exon 11 deletion MEFs (Brca1 Δ11) were compared to $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ MEFs. All results are means of at least three independent experiments and error bars show the standard deviation.

But, surprisingly, Brca1 $^{\Delta 11/\Delta 11}$ MEF showed considerable repair efficiency in ICL reporter reactivation assay (Fig. 3b). Although it is not the same level to $p53^{-/-}$ MEFs, this efficiency is comparable to Brca1-reconstituted MEFs. This result suggests that although the lost of large part of Brca1 protein which are important to DNA repair function, Brca1 $^{\Delta 11/\Delta 11}$ still have potential ability for ICL repair process at least in recombination-independent pathway.

Brca1-exon11 deletion MEFs exhibit high resistance to MMC

To confirm the data from poralen ICL reporter reactivation assay, I performed clonogenic survival assays against ICL agent MMC using $p53^{-/-}$, $p53^{-/-}Brca1^{-/-}$ MEFs, Brca1-reconstituted MEFs and Brca1 $^{\Delta 11/\Delta 11}$ MEFs. Consistent with previous report[4,8,9], $p53^{-/-}Brca1^{-/-}$ MEFs were hypersensitive to MMC treatment compare to $p53^{-/-}$ MEFs. In $p53^{-/-}$ $Brca1^{-/-}$ MEFs, cell survival rate was decreased sharply in a dose-dependent manner, while that of $p53^{-/-}$ MEFs was not changed significantly (Fig. 4).

Brca1-reconstituted MEFs showed comparable resistance to MMC treatment. The incomplete restoration of MMC resistance in Brca1-reconstituted MEFs could be due to undetected genomic alteration upon Brca1 gene integration. Although MMC resistance did not fully restored by retrovirus mediated Brca1 gene reintroduction, these results indicate that the hypersensitivity of $p53^{-/-}Brca1^{-/-}$ MEFs to MMC due to the absence of Brca1 function, and also

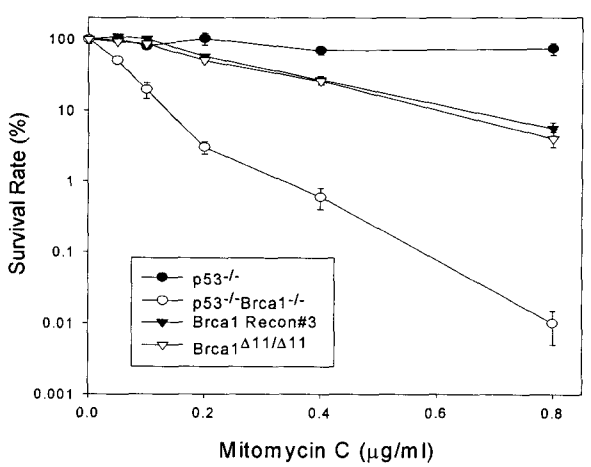

Fig. 4. MMC sensitivity in $p53^{-/-}$, $p53^{-/-}Brca1^{-/-}$ MEFs, Brca1-reconstituted cell line (R#3) and Brca1-exon 11 deletion MEFs (Brca1 $^{\Delta 11/\Delta 11}$). Clonogenic survival was examined in 100 mm dish after 1 hr treatment with various MMC doses. Colonies were stained 10-12 days after with methylene blue and counted. Error bars indicate the standard deviation.

suggesting that Brca1 may participates, directly or indirectly, in ICL DNA repair process through not only HR-dependent pathways but also recombination-independent manner.

Furthermore, consistent with previous data, I found that Brca1 $^{\Delta 11/\Delta 11}$ MEFs showed significant resistance than $p53^{-/-}Brca1^{-/-}$ MEFs (Fig. 4). Brca1 $^{\Delta 11/\Delta 11}$ MEFs showed almost the same level of resistance as Brca1-reconstituted MEFs at all doses of MMC treatment. Taken together, these results suggest that the exon 11 region is not abso-

lutely required in ICL repair function of Brca1 and Brca1 could be involved in recombination-independent ICL repair process through N-terminal RING finger or C-terminal BRCT domain instead of exon11 Rad50/51 binding domain.

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초록 : 재조합 비의존적 경로를 통한 DNA 사슬간 교차결합 복구에의 Brca1 단백질의 기능

윤 진 호*
(동아대학교 의과대학)

시스플래틴이나 마이토마이신 C (MMC)와 같은 DNA 사슬간 교차결합 (interstrand cross-link ; ICL) 물질에 대해 Brca1 결손세포들이 보이는 높은 감수성은 Brca1 단백질이 세포의 ICL 복구반응에 중요한 역할을 담당하고 있음을 암시하고 있다. Brca1 단백질은 재조합 의존성 또는 재조합 비의존성 경로를 통한 DNA 이중사슬 절단 (double-strand break ; DSB) 복구에 필수적인 역할을 담당한다. 최근 본인이 속한 연구그룹에서 재조합 의존성 경로를 통한 세포의 ICL 복구반응에 Brca1이 관여한다는 것을 밝혀 보고한바 있다. 본 연구에서는 Brca1 단백질의 재조합 비의존성 복구반응에 대한 관여여부를 $p53^{-/-}$ 와 $p53^{-/-}Brca1^{-/-}$ 세포주를 사용하여 연구하였다. 교차결합 복구 실험에서 Brca1 결손 세포주는 Brca1 정상 세포주보다 현저히 낮은 활성을 보였다. 또한, Brca1 결손세포주의 MMC 에 대한 감수성과 ICL 복구능이 Brca1 단백질 발현을 통해 회복되는 것을 확인하였다. 흥미롭게도, Brca1의 11번 엑손 결손세포주 (Brca1 △11)는 높은 MMC 저항성과 ICL 복구능을 보였다. 이러한 결과들을 종합하여 볼 때, Brca1 단백질은 ICL 복구에 재조합 의존성 경로 뿐만 아니라 재조합 비의존성 경로를 통해서도 관여하며, 이러한 활성에는 엑손 11 부분이 아닌 N 말단의 RING 핑거 도메인이나 C 말단의 BRCT 도메인이 중요하다는 것을 알 수 있다.