Abstract

BRCA1 is responsible for the hereditary breast and ovarian cancer. Although mounting evidence has indicated that BRCA1 participates in the cellular responses to repair DNA double-strand breaks (DSBs), its precise mechanism is still unclear. To elucidate the molecular function of BRCA1 as a tumor suppressor, we have analyzed the various functions of BRCA1, such as apoptosis, DNA repair, and cell cycle regulation.

Recently, we are analyzing the cellular response of BRCA1 to various DNA damage using molecular imaging technique. We examined in situ response of BRCA1 accumulation at DSBs produced by laser micro-irradiation. The amino (N) and the carboxyl (C)-terminal fragments of BRCA1 accumulated independently at DSBs with distinct kinetics. The N-terminal BRCA1 fragment accumulated immediately after laser-irradiation at DSBs. In contrast, the C-terminal fragment of BRCA1 accumulated more slowly at DSBs. Interestingly, rapid accumulation of the BRCA1 N-terminus, but not the C-terminus, at DSBs depended on the non-homologous end-joining (NHEJ) factor. Missense mutations found within the N-terminus of BRCA1 in cancers significantly reduced the accumulation at DSBs. These results suggest important roles of the N-terminus of BRCA1 with other repair factors at damaged sites influence its multifunctional roles in DNA repair.

On the other hand, BRCA1-deficient cells are sensitive to UV irradiation and BRCA1 localizes at nuclear foci after UV irradiation, although UV irradiation does not seem to induce DSBs directly. To analyze further the response of BRCA1 to UV irradiation, we generated local UV irradiation in cell nuclei using an isopore membrane filter. BRCA1 immediately accumulated at the site of local UV irradiation and the accumulation was abrogated by treatment cells with transcription inhibitors. Interestingly, the BRCA1 accumulation was dependent on the presence of the protein, which is involved in the nucleotide excision repair (NER) pathway. Furthermore, we found that BRCA1 is associated with this protein. These results suggest that BRCA1 is involved in TCR process for the DNA damage induced by UV irradiation.

Furthermore, we have identified new BRCA1 associated proteins by proteomic study and are examining the functions of those proteins. These precise analyses of BRCA1 and its related proteins will contribute to the development of novel molecular diagnosis and therapies for personalized medicine for breast and ovarian cancer.

1. Introduction

Approximately 5% of breast cancers show a familial pattern of occurrence. In 1990, genetic studies provided initial evidence that the risk of breast cancer in some families is linked to chromosome 17q21. In 1994, the breast-cancer susceptibility gene 1, BRCA1 was identified by positional cloning [1]. Our research has been focused on BRCA1. This gene encodes 1863 amino acids, and more than 200 different germline mutations associated with cancer susceptibility have been identified. Mutation carriers of BRCA1 also have increased susceptibility to ovarian cancers. The risk of breast cancer developing is about 50-80% and the risk of ovarian cancer is 12-60% in carrier of BRCA1 mutations. BRCA1-related breast cancers are characterized by early onset, a high frequency of bilateral involvement, and poor prognosis.

Somatic BRCA1 mutations are rarely observed in sporadic breast cancer. However, both BRCA1 mRNA and protein expression are down regulated in approximately 30% of sporadic breast cancer and 70% of ovarian cancers. It is believed that this may be due to non-mutational mechanism such as acquired methylation of the promoter or malfunctions in the upstream pathway that regulate BRCA1 expression. Therefore, lower expression or function of BRCA1 is thought be an important contributing factor in sporadic cancers.

BRCA1 contains a RING domain in the amino (N)-terminus and two tandem BRCT domains in the carboxy (C)-terminus. BRCT domain is frequently found in DNA repair proteins and function as a binding module for phospho-serine peptides. BRCA1 has also nuclear export signal (NES), nuclear localization signal (NLS), and DNA binding region. After DNA damage BRCA1 is phosphorylated by Chk2 or ATM (Fig. 1).
The N-terminal region of BRCA1 directly interacts with BARD1 and the association with BARD1 enhances the ubiquitin polymerase activity of BRCA1. Like BRCA1, BARD1 has a RING domain at its N-terminus and two BRCT domains at its C-terminus. BRCA1 has been implicated in a number of cellular processes, including the regulation of DNA damage repair, transcription, cell cycle, chromatin remodeling, and apoptosis.

To elucidate the molecular mechanisms of tumor suppressive function of BRCA1, we have been analyzed the various functions of BRCA1.

(1) Using biochemical technique, we resolved four distinct BRCA1-containing complexes. BRCA1 content of these complexes is altered following DNA damage and that one of these complexes, the HUIC (hydroxyurea induced complex), is associated with DNA damage. These suggest that BRCA1 participates in multiple cellular processes by forming multiple protein complexes [2].

(2) Furthermore, we found that BARD1, heterodimer partner of BRCA1, is an integral component of RNA polymerase II holoenzyme (holo-pol) and that the N-terminus of BRCA1 is important for the association with the holo-pol and the localization at nuclear foci in the S-phase of cell cycle [3].

(3) Expression of the amino terminus deleted BRCA1 revealed a powerful growth suppressive effect. Its growth suppression is associated with an increase in apoptosis [4].

(4) In addition, we identified RNA polymerase II (RNAPII) as a substrate of ubiquitination activity of BRCA1. After UV irradiation, subpopulation of RNAPII is phosphorylated and recognized by the C-terminus of BRCA1 as a substrate after UV irradiation. BRCA1/BARD1 ubiquitinate the phosphorylated RNAPII, which is subsequently degraded at the proteasome. Then, BRCA1/BARD1 recruits other DNA repair factor to the site of DNA damage. We presume that BRCA1 is involved in transcription coupled repair process by the mechanism shown in Figure 2 [5].

(5) Furthermore, we have developed the functional assay for the molecular diagnosis of BRCA1 by ubiquitination activity and subcellular localization in S-phase and after DNA damage.

Recently, our research has been focused on the DNA repair function of BRCA1. Among various function of BRCA1, the tumor suppressor activity of BRCA1 is to be primarily attributed to its involvement in DNA repair. Clinically observed missense mutations often result in non-functional BRCA1 proteins that lose the ability to repair DNA damage. However, the precise mechanism of what role BRCA1 plays in DNA repair pathway is not fully understood. Now, we are collaborating with Dr. Akira Yasui, Department of Molecular Genetics, Institute of Development, Aging and cancer, Tohoku University, and are analyzing the cellular response of BRCA1 to various DNA damage using molecular imaging technique.

2. Results and Discussion

2.1. BRCA1 response to various DNA damage induced by laser micro-irradiation

2.1.1. Laser micro-irradiation system

To elucidate the molecular mechanisms of DNA repair processes for various DNA damage of BRCA1, we are analyzing the BRCA1 accumulation at the DNA damaged site induced by laser micro-irradiation in living cells.

Our experimental system is a laser micro-irradiation apparatus combined with a confocal microscopy. We use a 365nm pulse laser system for the irradiation of cells in the epi-fluorescence path of the microscope system. A lower dose or higher dose of irradiation was obtained by passing lasers through distinct filters in front of the lens.
A 405nm pulse laser system is also used. The power of laser scan can be controlled by the number of scan (Fig. 3). By using these system, various types of DNA damage, such as single-strand breaks (SSBs), double-strand breaks (DSBs) and oxidative base damage, were produced at restricted nuclear regions of cells [6,7].

Fig. 3. Laser micro-irradiation systems. The left column shows the 365 nm pulse laser irradiation system, producing the lower dose and the higher dose irradiation, which are regulated by the filter in front of the mirror. The right column shows the 405 nm laser system. Fluorescent images were obtained and processed using an FV-500 confocal scanning laser microscopy system. A 405 nm scan laser system for irradiation of cells in the epifluorescence path of the microscope system was used. One scan of the laser light at full power delivers approximately 1600 nW. To primarily induce DSBs, we scanned cells 500 times using the 405 nm laser at full power, which has been shown The 405 nm laser light was focused through a 40× objective lens.

2.1.2. Endogenous BRCA1 accumulates at the laser micro-irradiated sites with different kinetics

Using this system, we found that BRCA1 accumulates at various DNA damage sites induced by laser-irradiation. Here, we show that different interaction of BRCA1 with repair factors at DSBs influence its multifunctional roles in DNA DSBs repair.

After DSBs were introduced into the nuclei of the cells by laser micro-irradiation, human Saos-2 cells were fixed and processed for immunofluorescence. Endogenous BRCA1 clearly accumulated at the irradiated sites (Fig. 4). Accumulation of phosphorylated H2AX (γH2AX), a protein that rapidly phosphorylated at the sites of DSBs after DNA damage was also observed. From the time course of accumulation of fluorescence intensity for each protein, we found that both the accumulation and clearance of BRCA1 were slower than that of γH2AX.

Fig. 4. Accumulation of endogenous and BRCA1 at the laser micro-irradiated sites. Immunohistochemical detection of BRCA1 in Saos-2 cells after 500 scans of 405 nm laser irradiation. Cells were fixed after laser irradiation and stained with antibodies against BRCA1.

2.1.3. GFP-BRCA1 accumulates at DNA DSBs

We next examined the real time localization of GFP-tagged BRCA1 in living cells after laser-irradiation. GFP-BRCA1 was transfected into Saos-2 cells and the cells were then laser-irradiated. As shown in Figure 5, GFP-BRCA1 clearly accumulated at the irradiated sites. The mean intensity of GFP-BRCA1 at the accumulation site was quantified. Accumulation of BRCA1 at the irradiated sites was gradual. The results indicate that the accumulation kinetics of overexpressed GFP-BRCA1 mimics that of endogenous BRCA1.

Fig. 5. GFP-tagged BRCA1 accumulates at DSB sites in Saos-2 cells.
2.1.4. BRCA1 localizes at DSBs via its N- and C-terminal regions

We next identified the regions in BRCA1 that mediate this accumulation. Several GFP-tagged deletion mutants of BRCA1 were constructed. The full-length BRCA1, as well as the deletion mutants, Δ305-770, Δ775-1292, Δ1-302, and Δ1527-1863, were transfected into Saos-2 cells. Protein expression was confirmed by Western blots using antibodies against BRCA1. Because the RING domain is located in a.a.1-302 and the BRCT domains are located in a.a.1528-1863, we hypothesized that deletion of either of these two regions would suppress BRCA1 accumulation at DSBs. However, each of the four deletion mutant BRCA1 proteins accumulated at DSBs. Therefore, the a.a.303-1526 BRCA1 fragment was constructed, which contained only the central region of BRCA1 and lacked both the N- and C-terminal regions. This BRCA1 fragment did not accumulate at the irradiated sites. These data suggest that either the N- or C-terminal region alone is sufficient for BRCA1 accumulation at DSBs. Subsequently, a.a.1-304 and a.a.1527-1863 fragments were constructed and each fragment was shown to accumulate at laser-induced linear DSBs.

The mean intensity of GFP-BRCA1 at the accumulation site was quantified and the kinetics of accumulation of full-length and these BRCA1 fragments were examined for 600 seconds after irradiation (Fig. 6). Interestingly, the N-terminal (a.a.1-304) fragment rapidly and maximally accumulated at the DSBs within 20 seconds, whereas the C-terminal (a.a.1527-1863) fragment slowly and gradually accumulated, reaching a plateau at 360 seconds. Full-length BRCA1 exhibited a pattern of accumulation that reflected the combination of N- and C-terminal fragment patterns.

The accumulation kinetics of the N-terminal BRCA1 was very fast, whereas endogenous BRCA1 accumulated at irradiated sites five minutes after irradiation. To examine whether the N-terminal BRCA1 accumulates at DSBs or at other sites of damage, we pre-treated cells with BrdU. The mean intensity of accumulation of the GFP-tagged N-terminus of BRCA1 was significantly enhanced by BrdU pre-treatment, indicating that the accumulation occurs at DSBs. Taken together, the above kinetic analyses suggest that the N- and C-terminal regions of BRCA1 may accumulate at DSBs through different mechanisms.

Next, we compared the clearance kinetics of the N- and C-terminal fragments of BRCA1 from DSBs. The accumulation of the N-terminal fragment along the line of irradiation was detected 10 min after laser-irradiation, but was almost completely lost by two hours after irradiation. In contrast, the fluorescence of the C-terminal fragment at DSBs was present at two hours after irradiation. These results indicate that the C-terminal region is unique in its ability to remain at the DSBs and that BRCA1 displays domain-specific kinetics in terms of its initial accumulation and subsequent retention at the damaged sites.

2.1.5. Accumulation of BRCA1 at DSBs requires the other DNA repair factor

Next, we examined whether BRCA1 accumulation at DSBs is dependent on specific DNA repair factors, using factor-deficient cells.

BRCA1 is phosphorylated by the Ataxia-telangiectasia mutated (ATM) kinase after ionizing radiation. GFP-BRCA1 N- and C-terminal fragments were transfected into human ATM-deficient AT1KY/T-n cells. The N- and C-terminal fragments accumulated at laser-induced DSBs in these cells. Thus, phosphorylation of BRCA1 by ATM is not required for BRCA1 accumulation at irradiated sites.

In H2AX-/- MEF cells, both N- and C-terminal fragments of BRCA1 accumulated at DSBs. Thus,
accumulation of BRCA1 is not likely to be dependent on H2AX, even though γH2AX was detected at the irradiated sites before BRCA1.

NHEJ and homologous recombination (HR) are the major cellular mechanisms to repair DSBs. In the NHEJ pathway, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and a Ku heterodimer of Ku80 and Ku70 are recruited to the sites of DNA DSBs, followed by subsequent recruitment of the XRCC4-ligase IV. In contrast, Rad51 and replication protein A (RPA) are the essential factors in the HR pathway. An Mre11-Rad50-Nbs1 complex is involved at an early stage of both HR and NHEJ pathways. Although BRCA1 is thought to be primarily involved in the HR pathway, it has been implicated in the NHEJ pathway as well.

In CHO-derived CHO9 XR-C1 (DNA-PKcs -/-) and XRV79B (Ku80 +/-) cells, the N- and C-terminal fragments of BRCA1 accumulated at the laser-irradiated sites. However, in XRV15B (Ku80 -/-) cells, the N-terminal fragment of BRCA1 failed to accumulate at DSB sites, whereas the C-terminal fragment did accumulate. Therefore, accumulation of the N-terminal fragment, but not the C-terminal fragment, of BRCA1 at DSBs is Ku80 dependent.

2.1.6. Missense mutations of BRCA1 reduce its Accumulation

We examined whether missense mutations would affect the accumulation of BRCA1 at DSBs. Four missense mutations were selected, on the basis of their relatively frequent occurrence in the Breast Cancer Information Core database (www.nhgri.nih.gov). However, the pathological significance of these mutations has not been verified.

GFP-BRCA1 constructs, each harboring one of the missense mutations, were generated. Each of the four mutants accumulated at the irradiated sites, but the fluorescence intensity was markedly diminished relative to that of the wild-type BRCA1 fragments (Fig. 7). All mutant constructs were expressed at levels similar to the wild-type BRCA1 fragments, as confirmed by Western-blot analysis. These accumulation kinetics suggest that these four missense mutations are likely to have a pathological significance.

2.1.7. Discussion

In this study, we laser micro-irradiated living cells and demonstrated that endogenous as well as GFP-BRCA1 accumulated at DSBs. Using various deletion mutants and fragments of BRCA1, we found that both the N- (a.a.1-304) and C- (a.a.1528-1863) terminal regions of BRCA1 accumulate at DSBs. Au et al. have reported that the RING and BRCT domains of BRCA1 cooperate in the localization of BRCA1 at irradiation-induced nuclear foci [8]. In those studies, the RING domain (a.a.1-304) did not form nuclear foci and the BRCT domain only poorly localized at foci, whereas an artificial fragment composed of the RING and BRCT domains localized at nuclear foci as efficiently as full-length BRCA1. One explanation for the apparent inconsistency between the previous report and our present findings is that the N-terminus of BRCA1 may not accumulate at nuclear foci, but only at DSBs. Alternatively, if the N-terminus of BRCA1 accumulates at DSBs, but remains at the damaged site only transiently, its fluorescence intensity at nuclear foci may not be detectable.

In fact, our detailed kinetic analyses revealed distinct behaviors of the N- and C-terminus of BRCA1. The N-terminal region of BRCA1 accumulated at DSBs immediately and transiently after laser-irradiation, whereas the C-terminal region slowly and gradually accumulated and remained associated with the DSB for longer times (Fig. 6). We propose that the N-terminal region may function to guide BRCA1 to the DSBs in the early phase of the repair process, and that the C-terminal region may be responsible for the accumulation in the later phase of repair and the sustained retention. Moreover, such a mechanism may apply to other DNA repair factors as well. Celeste et al. reported that in H2AX-/- cells, 53BP1 localized at irradiation-induced nuclear foci and laser-irradiated sites in the early phase, but not in the late phase of repair (in H2AX+/+ cells, 53BP1 accumulated in both the early and late phases) [9].

We analyzed the possible contribution of H2AX to BRCA1 accumulation at DSBs. Both N- and C-terminal
fragments of BRCA1 accumulated at DSBs, even in H2AX-/- cells. These observations are consistent with some reports that BRCA1 accumulates at laser-induced DSBs in H2AX-/- cells [9], but other studies reported that BRCA1 did not localize at nuclear foci in the same H2AX-/- cells [10, 11]. The requirement for H2AX may differ for BRCA1 localization at DSBs versus nuclear foci. Alternatively, Bassing et al. and Celeste et al. may have been unable to detect nuclear foci (note that their observations were made at 0.75-6 hours after irradiation) because the accumulation of BRCA1 at DSBs may be transient in the absence of H2AX. H2AX could be involved in retention of the C-terminal fragment of BRCA1, analogous to H2AX mediated 53BP1 retention in the late phase.

The N-terminus of BRCA1 accumulated at DSBs in a Ku80-dependent manner. Accordingly to Kim et al., other NHEJ factors, Ku70 and DNA-PKc, are only transiently localized to the laser-irradiated site [12]. In our analysis, the N-terminus of BRCA1 accumulated only transiently, suggesting that BRCA1 functions with NHEJ factors in the early phase of the repair process, and dissociates from the site of DSBs together with NHEJ factors.

Breast cancer risk has been correlated with single nucleotide polymorphisms in NHEJ factor genes. Furthermore, the BRCA1 genotype significantly affects the degree of this risk, if the NHEJ factors are a high-risk genotype. There have been conflicting reports on the role of BRCA1 in NHEJ, including promotion, suppression, or no effect. The existence of two subpathways have been recently proposed for NHEJ, namely error-free and error-prone NHEJ [13, 14]. Previously reports have indicated that BRCA1 functions to promote error-free NHEJ, or to inhibit error-prone NHEJ [15, 16]. It is therefore possible that the above facilitation and/or suppression of NHEJ might be achieved by the accumulating BRCA1 at DSBs.

The missense mutations that we analyzed significantly reduced BRCA1 accumulation at DSBs. The pathological significance of mutations found in the BRCA1 N-terminus have been evaluated only around the RING domain by examining BARD1 association and ubiquitination activity. In our analysis, those four mutants were able to interact with BARD1 and exhibited ubiquitination activity (unpublished data). We found for the first time that these four missense mutations could be pathological mutations since they disrupted BRCA1 accumulation at DSBs. These data suggest that rapid accumulation of BRCA1 at DSBs via its N-terminus is important its tumor suppressor activity.

Bekker-Jensen et al. reported that BRCA1 accumulates in two distinct nuclear compartments, the DSB-flanking chromatin marked by H2AX and ssDNA-microcompartments [17]. Down-regulation of the checkpoint mediator Mdc1/NFBD1 dissociates BRCA1 from the DSB-flanking chromatin, but not from ssDNA-microcompartments. This observation suggests that BRCA1 may form different types of protein complexes and that each complex may localize to distinct structures near sites of DNA damage. Our results indicate that BRCA1 forms distinct protein complexes using different intramolecular regions. Taken together, BRCA1 may contribute to multiple protein complexes, whose constitution is spatiotemporally and dynamically regulated, and which function in various phases of DNA DSB repair processes.

2.2. BRCA1 response to local UV irradiation

As mentioned above, in the field of DNA repair mounting evidences have indicated that BRCA1 is engaging in DNA DSBs repair pathways, HR and NHEJ. On the other hand, BRCA1 seems to be involved in the processes of transcription coupled repair (TCR) [18]. BRCA1-deficient cells are defective in a process in which oxidative base damage are removed preferentially from the transcribed DNA strand [19].

BRCA1 localizes in nuclear foci after UV irradiation. BRCA1 is phosphorylated on Ser1423 and Ser1524 by the ataxia telenguectasis mutated-related kinase (ATR) after UV damage. Previously we and other group reported that BRCA1 ubiquitinates RNAPII after UV irradiation [5,20,21].

The predominant DNA damage produced by UV irradiation is cyclobutane pyrimidine dimmers (CPD) and 6-4 photoproduct adducts. These lesions are removed by NER, which eliminates a wide variety of bulky helix-distorting DNA lesions. NER operate via two pathways: global genome repair (GGR) and TCR. GGR can repair DNA lesions at any location in the whole genome, while TCR selectively removes DNA lesions on the transcribed strands of expressed genes.

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Fig. 8. A schema of exposing cells to the local UV irradiation using membrane filter.

UV

filter

cells
To gain further insights into the regulation of BRCA1 upon TCR pathway, we induced UV damage in restricted small areas of the cell nucleus by an isopore membrane filter. Cells mono-layered in 35-mm glass-bottom dishes were treated with or without transcription inhibitor before being covered with a polycarbonate isopore membrane filter with pores of 3μm in diameter and 254nm UV irradiated with a dose of 60J/m² (Fig. 8). The polycarbonate blocked the 254nm UV-light, and cells were exposed only though the pore of the filter and immunostained with antibody against CPD and BRCA1. After local UV irradiation, CPD, the major types of DNA damage induced by UV irradiation, was observed as fine nuclear dots and BRCA1 colocalized with CPD in the cell nucleus. When cells were stained with another antibody against BRCA1, C-20 (Santa Cruz), we found similar images of BRCA1 accumulation. These suggest that BRCA1 immediately accumulates at the UV damaged sites (Fig. 9).

To examine whether the BRCA1 accumulation at the sites of local UV irradiation is associated with transcription, similarly as localization at nuclear foci, we treated the cells with actinomycin D or ρ-Amanitin before exposing to local UV irradiation. Treatment with these chemical completely abolished the accumulation of BRCA1.

Furthermore, we found that the BRCA1 accumulation at the UV irradiated sites was dependent on the presence of the protein, which is involved in the NER pathway and that BRCA1 is associated with the protein. Our data showed the mechanism of involvement of BRCA1 in the TCR at the lesion induced by UV irradiation.

2.3. Identification of BRCA1 associated molecules

We identified new some BRCA1 associated proteins by proteomic study. Now we are examining their roles in BRCA1 function, such as ubiquitination activity, DNA repair pathway, and the effect to drug sensitivity.

3. Conclusion

Recently, it is reported that inhibition of poly (ADP-ribose) polymerase 1 (PARP1), which is a critical enzyme to SSB repair pathway, leads to severe, highly selective toxicity in BRCA1-deficient cells [22]. This seems to be because the inhibition of PARP results in unrepaired SSBs, giving rise to DSBs. These represent a new concept in cancer treatment. Lower expression or function of BRCA1 is also observed in sporadic cancers, suggesting that precise analysis of BRCA1 and its associated protein will contribute to the discovery of novel molecular target of cancer chemotherapy.

In cancer treatment, it is important to select the treatment method and predict its effectiveness in individual cancer patients. Molecular targeting therapies are focuses of investigation with a view to developing novel approach to cancer control. New biomarkers are similarly viewed as essential for improvement of cancer diagnostics. Our research is focused on identification and functional analysis of the critical proteins that play a key role in the carcinogenesis, and development of the biomarker of diagnosis and molecular targets for therapies for personalized medicine for breast and ovarian cancer. Molecular imaging technique will be a useful tool for those aims.

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