Biological Significance of DNA Damage Checkpoint and the Mode of Checkpoint Signal Amplification

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It is generally accepted that DNA damage checkpoint is the mechanism that allows time for DNA damage repair. However, several lines of evidence challenge this paradigm, especially, in the case of G1 checkpoint. The first evidence is the complete difference between the repair kinetics of DNA double-strand breaks (very rapid) and the timing of G1 checkpoint induction (very slow) after ionizing radiation. The second evidence is that inactivation of p53, which is a central player of G1 checkpoint, does not render cells radiosensitive, rather, such cells become radioresistant. Moreover, it was shown that G1 arrest persists almost permanently after irradiation, until the time when most of the initial damage should be repaired and disappear. Therefore, cells should have a mechanism to maintain G1 checkpoint signaling by amplifying the signal from a limited number of damage. In this review, we discuss what is the *bona fide* role of G1 arrest and how G1 checkpoint signal is maintained long after irradiation.

Biological significance of DNA damage checkpoint

DNA damage-induced cell cycle checkpoint is a finely-tuned mechanism, in which many factors are well coordinated. The conductor of the cell cycle checkpoint is ATM protein kinase, which orchestrates all checkpoints, namely, G1, intra-S, and G2/M checkpoint.[1] ATM protein is a responsible gene product for the congenital cancer-predisposed disease, ataxia-telangiectasia (AT).[2] Cells derived from AT patients show hyper-radiosensitivity, have a defect in DNA double-strand break repair, and exhibit defective DNA damage checkpoints, including G1, intra-S, and G2/M arrest.[1,3-5] ATM protein belongs to phosphatidylinositol 3-kinase-like-kinase family, and it phosphorylates many checkpoint-, or repair factors, such as p53 (Ser15), CHK2/hCds1 (Thr68), and NBS1 (Ser278 and 343).[6-12] Recently, the mechanism of ATM activation in response to DNA double-strand breaks was reported.[13] Although ATM forms dimer or higher-order multimer in unperturbed cells, it is activated through intermolecular autophosphorylation at its Ser1981 and monomerization. The Ser1981-phosphorylated ATM molecules assemble at specific sites in the nucleus after irradiation, which can be visualized as "foci" by immunofluorescence staining.[13] Each radiation-induced focus of the phosphorylated ATM likely represents each DNA double-strand break, because the number of foci after X-rays is consistent with the number of DNA double-strand breaks induced by the dose of X-rays physically calculated, like the number of Ser139-phosphorylated histone H2AX foci, which colocalize with the phosphorylated ATM foci.[14-16]

Much earlier than the discovery of ATM protein, cell cycle delay, which is later called cell cycle checkpoint, in DNA damaged eukaryotic cells was reported in G2-M transition in 1956.[17] Thereafter, several groups studied the role of G2 arrest using premature chromosome condensation method by fusion with mitotic cells or by treatment with caffeine, and following results were obtained: Breaks were revealed in the condensed chromosomes in cells that had been forced prematurely from the DNA damage-induced G2 arrest, while fewer breaks were observed in chromosomes either forced to condense after a G2 delay or permitted to progress naturally to mitosis.[18-20] Moreover, cell viability was lower if DNA damaged cells were forced to escape from G2 arrest by treatment with caffeine, which is now shown to inhibit ATM/ATR, than when cells were mock-treated.[21] From these results, it became generally accepted that DNA damage is repaired during G2 arrest, that cell division in the presence of chromosome damage is lethal, and therefore, that G2 arrest is indispensable for viability of DNA damaged cells. In 1989, decisive data were re-

ported by Weinert and Hartwell.[22] In this paper, they searched a budding yeast strain which is defective in X-ray-induced G2 delay from radiation sensitive (RAD) mutants, and found that *rad9* cells failed to undergo G2 arrest. Moreover, they found that efficient DNA repair can occur in irradiated *rad9* cells if irradiated cells are blocked for several hours in G2 by treatment with a microtubule poison. Their study revealed that G2 arrest is controlled by molecular mechanism, rather than by structural constaints of the damaged DNA that directly prevent entry into mitosis.

Since G2 arrest is induced rapidly (within 1 h after irradiation), it might be reasonable to consider that G2 arrest play a role in providing time for DNA double-strand break repair, which occurs very rapidly. Half-life of DNA double-strand breaks after 10-80 Gy of X-rays in normal human fibroblasts is less than 2 h.[23] In contrast to G2 arrest, G1 arrest is induced much more slowly, which becomes evident 6-8 h after irradiation, probably because this arrest requires p53-dependent gene expression, such as *p21*.[24,25] Therefore, most of the initial DNA double-strand breaks are already repaired at the onset of G1 arrest. Furthermore, it was demonstrated that ionizing radiation-induced G1 arrest persists almost permanently in normal human fibroblasts and the G1-arrested cells show a senescent phenotype, such as senescence-associated ß-galactosidase staining.[25,26] And, although cells would become radiation-sensitive when a G1 arrest factor is inactivated if G1 arrest allowed time for DNA repair, cells become radiation-resistant when p53, which is a key player of G1 arrest, is inactivated.[27] These lines of evidence strongly suggest that G1 arrest is the mechanism that permanently suppresses propagation of cells with irreparable DNA damage, rather than that spares time for DNA repair.

Amplification of G1 checkpoint signals

Then, how cells maintain persistent G1 arrest when most of the initial DNA damage disappeared? Since DNA damage is the source of checkpoint signaling, signals from a limited number of residual damage must be amplified sufficiently to maintain G1 checkpoint signaling. Recently, we revealed how cells achieve it.[28]

To demonstrate the existence of such "signal amplification mechanism", we focused on ATM protein kinase, which is a master regulator of DNA damage checkpoint as described above. We speculated that the nuclear foci of the phosphorylated ATM grasp a key to clarify the amplification mechanism of checkpoint signals. We first performed temporal analysis of the phosphorylated ATM foci after 1 Gy of X-rays in normal human diploid cells. Foci formation was observed immediately after irradiation and the number of foci peaked at 15 min after irradiation (36.9), and then, decreased time-dependently. Twenty-four hours after irradiation, the average number of foci per nucleus dropped to 1.8. However, interestingly, we found that remaining foci seemed to grow (increase in size) from 4 h after irradiation, compared to the foci observed 30 min after irradiation. The mean foci diameter becomes 0.8 µm at 4 h, while it is 0.5 µm at 30 min. The foci size increased up to 24 h after irradiation, and the

mean foci diameter reached 2.0 µm at this time. The similar growth of the remaining foci was also observed for other checkpoint-, or repair factors, such as Ser139-phosphorylated histone H2AX, MDC1, 53BP1, and NBS1, of which foci colocalized perfectly with the phosphorylated ATM foci. We also found that the growth of the residual 53BP1 foci is severely compromised in primary fibroblasts derived from AT patients (AT2KY and AT5BI) and a Nijmegen Breakage Syndrome patient (WG1799). Especially, 1.6 µm foci were rarely found in these cells.

We speculated that this foci growth might be the amplification mechanism of checkpoint signals, and therefore, examined the relationship between the foci growth of the phosphorylated ATM and G1 arrest. G0-synchronized normal human diploid cells were irradiated with 1 Gy of X-rays, then, replated at low density to release from synchronization. Twenty-four hours after release, cells were fixed and subjected to immunofluorescence staining for phosphorylated ATM and replication protein A (RPA), which is a marker for S phase entry. In this analysis, we categorized focus status in cells into three groups: without a focus, a small focus (<1.6 µm), and a large focus (1.6 µm). Intriguingly, the large foci were rarely (0.9%) observed in S phase progressed cells, while the small foci were frequently (45.9%) found in the S phase cells. The grown foci of phosphorylated ATM persisted at least 96 h after irradiation, suggesting grown foci persist permanently in G1-arrested cells. We next assessed the involvement of the foci growth in G1 checkpoint signals, by examining the relationship between the focus size of the phosphorylated ATM and the level of p53 phosphorylation at Ser15. In this experiment, we used G0-synchronized normal human diploid cells to exclude the possibility of p53 phosphorylation by ATR in S-, or G2 phase. And more, we treated cells with Nutlin-3, which stabilizes p53 by inhibition of p53-MDM2 binding, to make p53 levels constant in all cells.[29] We analyzed cells with one focus 24 h after irradiation, and found strong correlation between the focus diameter of phosphorylated ATM and the level of p53 phosphorylation (R=0.705). These results indicate that single irreparable damage can be recognized and signaled, and the signal can be amplified sufficiently to maintain G1 checkpoint signaling.

G1 checkpoint, potentially lethal damage repair, and chromatin structure

As described above, we would like to propose that the biological significance of G1 checkpoint is not sparing time for DNA repair, but the elimination of cells with irreparable DNA damage and cells have a mechanism to amplify G1 checkpoint signals from such a limited number of irreparable damage. However, there is a phenomenon, based on which one might argue for the "allowing time for repair" role of G1 arrest. That is "potentially lethal damage repair (PLDR)", which is evidenced by the increased cell survival when irradiated G0-synchronized cells are kept stationary for several hours before release from synchronization, instead of immediate release.[30] However, we don't think the PLDR is the evidence that G1 arrest

allows time for repair, because holding cells stationary is artificial, and cells should initiate G1 checkpoint signaling irrespective of release timing. There is sufficient time for G1 checkpoint induction even when G0-synchronized cells are released immediately after irradiation, because S phase entry of G0-synchronized normal human primary cells requires more than 12 h after synchronization release (our observation). Rather, the PLDR may indicate that checkpoint signal from the potentially lethal damage cannot be amplified sufficiently for G1 checkpoint activation, but the potentially lethal damage can be repaired during artificial G1 arrest. In other words, G1 checkpoint may not be activated in cells with such potentially lethal damage. If this were true, defective PLDR in AT cells would indicate only DNA-repair function of ATM contribute to the PLDR, though ATM plays a dual role in checkpoint and DNA repair. Recently, it was reported that phosphorylation of histone H2AX does not occur efficiently in heterochromatin region, though the mechanism remains unknown.[31,32] And in our pilot experiment, localization of the large phosphorylated ATM foci and heterochromatin region represented by heterochromatin protein 1α (HP1α) or CENP-A (centromere-binding protein) seem mutually exclusive. Taking these results into account, it is interesting to speculate that the potentially lethal damage may exist in heterochromatin, and thus, foci formation of phosphorylated ATM and phosphorylated H2AX does not occur, which results in defective checkpoint activation. It may require more time to repair the damage in heterochromatin than to repair the damage in euchromatin, probably because local chromatin decondensation by some unknown mechanism might be necessary for the access of repair factors.[33]

Conclusion

As discussed above, we think we are facing the necessity of paradigm shift concerining the role of G1 checkpoint from "allowing time for repair" to "terminating proliferation of cells with irreparable damage". We believe such paradigm shift would contribute to the better understanding of the molecular mechanism underlying G1 checkpoint.

Acknowledgements

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