Radiation Induced Dynamic Mutations and Transgenerational Effects

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Many studies have confirmed that radiation can induce genomic instability in whole body systems. Although the molecular mechanisms underlying induced genomic instability are not known at present, this interesting phenomenon could be the manifestation of a cellular fail-safe system in which fidelity of repair and replication is down-regulated to tolerate DNA damage. Two features of genomic instability namely, delayed mutation and untargeted mutation, require two mechanisms of ‘damage memory’ and ‘damage sensing, signal transduction and execution’ to induce mutations at a non damaged-site. In this report, the phenomenon of transgenerational genomic instability and possible mechanisms are discussed using mouse data collected in our laboratory as the main bases.

DYNAMIC MUTATIONS

The term dynamic mutation was originally coined to describe the expansion of the trinucleotide repeats sequences associated with human neuromuscular degenerative disorders. Patients with these disorders carry mutated allele of the target gene which has a higher than normal number of tandem trinucleotide repeat. The trinucleotide repeat of the affected allele further changes its copy number in somatic and germ cells. The precise mechanism of trinucleotide expansion in somatic and germ cells is not known at present, but the dynamic nature of the expansion poses a challenge to the current concept of mutagenesis in which mutation is thought to arise as the result of misrepair and misreplication of DNA damage. In the case of trinucleotide expansion, there seems to be no requirement of DNA damage at the alleles. Rather, the sequence of the affected allele itself is the cause of the expansion. In fact, the boundary length of normal and affected alleles is reported to that of the Okazaki fragment. DNA replication along the entire genome is not a uniform process and the regional sequence context together with the local chromatin configuration affect the replication fork progression, the fidelity of polymerization and the rate of recombination. Thus, mutation can arise as a non targeted event.

Dynamic nature of mutagenesis is not restricted to trinucleotide repeat expansion and in fact, the phenomenon was already observed in the 1940s in chemical mutagenesis research. In her pioneering work on the effect of mustard gas to Drosophila, Auerbach demonstrated that treatment of parental males (P₀ generation) with this chemical induced sex-linked recessive lethal in the F1 progeny (scored in the F2), but also in the F3 generation (She called them ‘replicating instability’). Mustard gas is an alkylating agent and the resulting DNA damage can be repaired within a few hours in the treated fly. Therefore, one has to conclude that the delayed mutation occurred even after three generations of reproduction and the treatment induced a higher mutability which persisted through generations even without DNA damage. Similarly, the classic work of Nomura clearly showed that parental irradiation increased the frequency of lung adenoma in F1 mice. The increase was observed in progeny derived from irradiated spermatozoa, spermatid and spermatogonial stages. The magnitude of observed increases, however, are difficult to reconcile with the known induced germine mutation rates in mice. For example, the frequency of adenomas in the progeny derived from irradiated spermatozoa (5 Gy) is around 20% addition, and the figures are approximately similar in the progeny derived from the other irradiated germ cell stages. When the spontaneous frequency of 5% is taken into account, the induced rate of adenomas is roughly 3% per Gy. This is three orders of magnitude higher than the estimated germ cell mutation rate of $3 \times 10^{-5}$/locus/Gy in specific locus experiments.

This simple comparison of the rates indicates that the mechanism responsible for the F1 tumors cannot be those induced directly by radiation at tumour related genes in gonad of parents. Similar to the delayed mutation of Auerbach, tumour causing mutation could have arisen in somatic cells of
F1 mice born to irradiated parents.

**MUTAGENESIS AS AN ACTIVE RESPONSE OF CELLS**

The above examples of delayed and high frequency mutations suggest an interesting possibility that some mutation may occur not as a passive consequence of DNA damage, but as a result of active cellular response to DNA damage. Although genomic integrity is crucial for somatic cells, the fidelity is rather costly since it requires precision in repair and replication. Recent studies have revealed that at least *E. coli* cells prefer down-regulating the fidelity of replication and repair over precision. This damage tolerance allows damaged cells to survive and proliferate at the cost of possible mutation. Thus, damage tolerance is a mutagenic cellular response to genotoxic stress.

The active mutagenic pathway of cells can be triggered not only by DNA, but by a wide variety of stresses. In fact, it has been known for more than a decade that mutation rate can be augmented by culturing *E. coli* cells in nutritionally poor media. This phenomenon was termed later as ‘adaptive mutation’ and was thought to be the mechanism used by bacteria to adapt to the harsh environment more rapidly by increasing the mutation rate. Adaptive mutation is not confined to bacteria and can be found in nutritionally-deprived yeast cells. However, it is not known whether a similar adaptive mechanism is present in mammalian cells.

**RADIATION-INDUCED GENOMIC INSTABILITY**

Recent studies with irradiated tissue culture cells have provided evidence for the induction of dynamic mutations in mammalian cells as well. Alpha-particle irradiated mouse hematopoietic stem cells were found to produce chromosome aberrations even after many cycles of replication. Similar chromosomal instability has also been observed in a variety of cell types, but the mechanisms remain to be elucidated. In addition to chromosomal instability, radiation can also induce another type of instability which produces gene mutations. These delayed instabilities are now generally termed as genomic instability.

Mutation induction by genomic instability has two characteristic features: untargeted mutation and delayed mutation (Fig. 1). Untargeted mutation requires the operation of ‘damage sensor, signal transducer and effector’ to induce mutation at non damaged sites. Delayed mutation requires ‘damage memory keeper’ to upregulate the untargeted mutagenesis system.

Untargeted and delayed mutations arising as a result of radiation-induced genomic instability, if they persist through generation, can explain the puzzling observations of trans-generational carcinogenesis in Nomura’s mouse experiments. Additionally, the possible involvement of delayed genomic instability in radiation carcinogenesis is of particular interest. The data of solid cancers in A-bomb survivors show a linear dose dependent increase in frequency, suggesting single-hit kinetics of cancer induction by radiation. However, this appears inconsistent with the well-accepted mechanism of multi-step carcinogenesis. Furthermore, solid cancer develops after a long latency period of a few decades after radiation exposures. This raises the question of whether or not the relevant carcinogenic mutation is induced in a delayed manner as a result of genomic instability, instead of being induced by the direct mechanism, although there exists a model in which direct mutagenic action of radiation can be sufficient for later development of cancer.

**TRANSGENERATIONAL MINISATELLITE MUTATIONS IN MICE**

The induction by radiation of genomic instability in mouse germ cells *in vivo* has been studied using hypervariable minisatellite sequences. Minisatellite sequences are composed of a stretch of short tandem repeats which were originally discovered in human genome. These sequences show high spontaneous mutation rates in germ cells and to a lesser extent in somatic cells. The mutational changes manifest as changes in the number of tandem repeat cores, and hence allele length. Thee postulated mutational mechanisms include slippage during replication, intra-allelic recombination, unequal sister chromatid exchanges or to simple deletion. Mouse minisatellites have shorter repeat units than those of humans with no sequence variation within a stretch. Therefore, mouse minisatellites have been renamed as expanded simple tandem repeats (ESTRs). In our work, we used Ms6hm, a hypervariable ESTR sequence as a marker for studying germ cell mutations. The Ms6hm locus is 3 to 10 kb in length with a short GGGCA repeat and is highly variable among laboratory strains of mice as well.

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**Fig. 1.** A scheme of genomic instability with two features of untargeted mutation and delayed mutation.
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as among individuals of the same strain.\(^7,8\) The high mutability of the locus facilitates the analysis of mutations using small sample sizes. Besides, the strain differences permit one to identify the parental origin of the mutation in the F1 progeny derived from a cross between two strains.

Evidence that the progeny of irradiated males showed higher frequencies of mutations at the paternally inherited Ms6hm locus has been published.\(^9\) While all germ cells stages respond to mutation induction, spermatids are the most sensitive. In a strict sense, mutation induction in the spermatozoan stage may not be considered as male germ line events since spermatozoa lack biochemical activity and mutation fixation takes place in fertilized zygotes. However, introduction of DNA damage into the egg via the sperm permits studies of untargeted events, namely, mutations in the non-irradiated maternal allele as discussed below.

In these experiments,\(^20\) male (C56BL/6N) mice were X-irradiated (6 Gy) and immediately mated to unirradiated (C3H/HeN) female mice and the F1 progeny descended from irradiated spermatozoa were used for the analysis. The results showed that the mutation frequencies were increased not only in the paternal allele (as expected), but also in the maternally-derived allele. Mutations in the maternal allele clearly demonstrates the untargeted nature of mutation induction in this system. The inference that mutation induction at the paternal allele is also untargeted rests on the following arguments: (a) a dose of 6 Gy induces about 300 DNA DSBs; (b) the Ms6hm sequence in the C57BL strain is about 10 kb; (c) the chance that a mutation will occur as a result of direct damage to the locus is about \(10^{-3}\) and (d) the observed increase in mutation frequency is of the order of \(10^{-3}\) and this is two orders of magnitude higher than expected.

It is important to mention here that the F1 progeny descended from irradiated spermatozoa provide no evidence for induced mutations at the maternal allele. This observation can be explained under the assumption that in these stages, the repair of radiation-induced DNA damage is already completed prior to their progression through the remaining stages of spermatogenesis and that the ‘damage factors’ which cause destabilization in the fertilized egg causing untargeted mutations in the maternal allele are no longer present (unlike the situation when irradiated spermatozoa are used for fertilization).

**TRANSGENERATIONAL MUTATIONS AT THE PINK-EYED UNSTABLE ALLELE LOCUS IN MICE**

The pink-eyed dilution locus has several mutant alleles. The pink-eyed unstable allele \((p-un)\) has a partial tandem duplication which reverts somatically to the wild type at high frequencies.\(^21\) Somatic reversion mutation can easily be scored in the retinal pigment epithelium (RPE) as clusters of black pigmented cells. The pink-eyed Jackson allele \((p-J)\) on the other hand is due to a deletion, and therefore no reversion takes place for this allele. The combination of these two alleles offers yet another tool to study delayed transgenerational reversion mutation induced by radiation. Male mice homozygous for the \(p-J\) allele were irradiated with 6 Gy of X-rays and immediately mated with the female homozygous for the \(p-un\) allele. The F1 progeny were analyzed for the reversions at the maternally inherited \(p-un\) allele.

The data show that the reversion frequency is around 3 to 5 spots per RPE in the unirradiated control and 7 to 8 in the irradiated group.\(^22\) Since the RPE develops at day 11 to 12 in the fetus, induced mutations in this system are delayed events occurring after many cycles of replication following the introduction of damage (via sperm) the egg. As in the case of the ESTR mutation, F1 mice descended from irradiated spermatocytes or spermatogonia showed no evidence for induction of mutations at the maternally-inherited \(p-un\) allele.

Interestingly, the induced (but not spontaneous) reversion of the \(p-un\) allele was found to be \(p53\) dependent, since no increase in the reversion was observed in \(p53^{-/-}\) F1 mice (Shiraishi, manuscript in preparation).

**GENOMIC CROSS-TALK AND P53-DEPENDENT S PHASE CHECKPOINT IN EARLY MOUSE EMBRYOGENESIS**

The observations on untargeted and delayed mutations of the maternal allele in the F1 progeny descended from irradiated sperm support our hypothesis that damage sensing/transducer/effecter system and a damage memory-keeping system must exist (Fig. 1); the first of these senses the DNA damage in the male genome and sends a signal to the female genome. This ‘genomic cross-talk’ results in untargeted mutation in the latter.

Mouse zygotes are known to possess a high level of p53 protein. Since at this stage, sperm and oocyte genomes exist as separate pronuclei in which one round of DNA synthesis occurs prior to the first cleavage division, we tested the ‘pronuclear cross-talk hypothesis’ by microinjecting a reporter with the p53 responsible promoter into the female pronucleus of the zygote and examined its activation by irradiated sperm. Clear evidence for such cross-pronuclear activation of the reporter was observed.\(^23\)

In the same experiment as above, we studied the S-phase progression by pulse-labeling with \(^3\)H-thymidine of control zygotes and zygotes fertilized with 6 Gy irradiated sperm (sperm irradiated zygotes). In both groups, pronuclear DNA synthesis was first detected at 8 h after fertilization and the first cleavage division occurred 23 to 24 h after fertilization. This observation suggests that the p53-dependent G1/S and G2/M checkpoints do not operate in the zygotes.
the amount of $^3$H-thymidine uptake was severely suppressed by sperm irradiation. Interestingly, DNA synthesis was suppressed also in the female pronucleus to a similar extent as in the male pronucleus. The extent of suppression suggested that the p53 dependent S checkpoint operates in a low dose region up to 2 Gy to sperm. Also, this suppression of DNA synthesis was not observed for sperm irradiated p53$^{-/-}$ zygotes.

We are not aware of any study in which evidence for a role of p53 in S-phase checkpoint has been found and our observation is the first one to suggest this novel function. This p53 dependency was also observed in primary mouse fibroblasts. One of the reasons why previous studies were unable to detect the p53 dependency could be that this novel S checkpoint seems to operate only in the low dose range of below 2 Gy while most of the S checkpoint studies have used much higher doses of 10 to 20 Gy.

Further analysis of sperm irradiated p53$^{-/-}$ zygotes indicated that the suppression could be restored by microinjection of p53 protein and this was further exploited for the analysis of the functional domain of p53 protein for this novel S checkpoint function (Fig. 2). Mutation in the DNA binding domain, but not in the transactivation domain was found to abrogate the activity, demonstrating the importance of the domain for S-checkpoint. The mechanism of the suppression of DNA synthesis was studied by the IdUrd and ClUrd double labeling method in mouse embryonic fibroblasts of p53 wild type and the p53$^{-/-}$ genotype. The results indicated that the speed of replication fork progression was slowed down in the wild type cells after exposure to 1–2 Gy but the replication origin firing was not affected. Further analysis has shown that the p53 function in S checkpoint is located downstream of ATM kinase which is required for phosphorylation of yet to be identified target protein needed for the p53 dependent suppression. The preliminary working hypothesis that we have developed for p53 dependent S-phase checkpoint is shown in Fig. 3. In this model, DNA damage sensor detects and sends signals to ATM kinase which with the help of p53 phosphorylates the third protein which then slows down the progression of the replication fork.

**P53-DEPENDENT ENHANCEMENT OF RECOMBINATION BETWEEN SISTER CHROMATIDS**

The above observations demonstrate that p53 is involved in retarding the replication fork progression after irradiation.

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**Fig. 2.** Restoration of the Suppression of DNA synthesis in sperm irradiated p53$^{-/-}$ zygotes by microinjection of p53 protein.

**Fig. 3.** A model of p53 mediated slowing down of replication fork progression after irradiation.
This retardation is an untargeted event since we have already shown that DNA synthesis of female pronuclei was suppressed in sperm irradiated zygotes. Since our results of untargeted and delayed mutation at the minisatellite locus and the p-un allele of the whole body system are all due to recombination, we have studied if the p53 dependent S checkpoint has any effect on recombinational events. In somatic cells, recombination occurs between sister chromatids and between homologous chromosomes, the latter being much less frequent in normal cells but highly frequent for some tumor cells.

We compared the frequencies of radiation-induced sister chromatid exchanges (SCE) in p53+/− and p53−/− mouse fibroblasts. Irradiation of the fibroblasts increased the frequency of SCE in a dose dependent manner in p53 wild type fibroblasts. To our surprise and excitement, this increase was not observed when p53−/− fibroblasts were examined, suggesting that radiation induction of SCE required the functional p53 (Toyoshima and Niwa, unpublished observation).

A POSSIBLE MECHANISM FOR UNTARGETED RECOMBINATION

Our results presented in this report suggest that p53 slows down the replication fork progression upon detection of radiation damage and the slow movement of replication fork is likely to increase the chance of recombination between sister chromatids. This p53-dependent elevation of recombination is the likely reason for the untargeted mutation observed in our study of transgenerational instability of the minisatellite and p-un alleles. Should this turn out to be correct, the resulting recombinational mutations at these two marker loci represent the error-free type exchange events and not the error-prone events such as delayed chromosome instability and genomic instability.

Minisatellite sequences undergo dynamic mutations in human and mice. This type of mutation has been the subject of intensive studies since it was thought to risk-related events such as induced genomic instability. In some reports, minisatellite instability was transmitted through two generations in the descendants of irradiated male parents. Preliminary results of studies with the p-un system suggest that somatic instability of this loci persists only for the generation born to irradiated males and the mutation frequency in the next generation decreased to the control level (Shiraishi, manuscript in preparation). In addition, chromosome instability was not observed in the atomic bomb survivors exposed in utero. Thus, some of the instability markers such as the mouse Ms6bm locus and the p-un allele of the pink-eyed dilution locus may not be suitable for the study of risk-related delayed chromosome instability and genomic instability induced by radiation. It is also likely that embryogenesis and fetal development may be well protected from radiation induction of delayed chromosome instability and genomic instability, except in rare mutant mice.

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