Low Dose Radiation Induced Genome Instability.

There are many different model systems that have been used to study chromosome instability. What is clear from all these studies is that conclusions concerning chromosome instability depend greatly on the model system and instability endpoint that is studied. The model system for our studies was the human B-lymphoblastoid cell line TK6. TK6 was isolated from a spontaneously immortalized lymphoblast culture. Thus there was no outside genetic manipulation used to immortalize them. TK6 is a relatively stable p53-normal immortal cell line (37). It shows low gene and chromosome mutation frequencies (19;28;31). Our general approach to studying instability in TK6 cells has been to isolate individual clones and analyze gene and chromosome mutation frequencies in each. This approach maximizes the possibility of detecting low frequency events that might be selected against in mass cultures.

Studies at the chromosomal level

Baseline levels of chromosome mutations were determined for individual and independently-derived clones of TK6. There was wide variation in chromatid break frequencies, but the average break frequency measured in a subset of clones at 0.04 ± 0.01 breaks/cell was only slightly higher than that reported for human peripheral blood lymphocytes. In contrast, dicentric frequencies at 0.006/cell were ten-fold higher for TK6 cells than that reported for human blood cells. The dicentric chromosomes observed in TK6 are unusual in that, unlike most mutagen-induced dicentrics, they had no associated acentric fragment. Thus these dicentrics appear to be formed from end-to-end fusions between chromosomes. Most of the fusions were between two different chromosomes, but small numbers of fusions between two homologues and between the two arms of the same chromosome (centric rings) were also observed. TK6 cells also showed higher levels of non-reciprocal chromosome translocations than observed in human lymphocytes. The results are consistent with a defect in telomere metabolism in TK6 cells that leads to telomere fusions and dicentric formation. Viable resolution of these dicentrics preferentially leads to nonreciprocal translocations, and any genetic alterations that occur as a result of dicentric formation and resolution in TK6 cells must involve only those sequences at or very near telomere sequences.

We reported on the effect of IR exposure on the frequency of clones containing cells with dicentrics (29). TK6 was exposed to different doses of IR and then surviving clones isolated and expanded for chromosome analysis. At the time of analysis, individual cells had undergone at least 30 cell division cycles. Instability levels were dramatically increased in exposed cells. There was about a 10-fold increase in the frequency of dicentrics/cell. A 5 cGy dose increased chromosome fusion frequency in TK6 clones from 0.002 ± 0.001 to 0.019 ± 0.001 (29). (Note that some of these frequencies differ from earlier reports, as we used a different clone of TK6 cells to reduce the likelihood that our original observations on instability in TK6 cells reflected the use of an unusual clone of TK6 cells.) There is no obvious dose-dependence for radiation-induced

instability in TK6 cells. Beyond the 5 cGy dose of γ-rays, there was no further increase in dicentric levels in TK6 cells. Instability levels do not increase with exposures of 10-400 cGy.

Influence of p53: Chromosome fusion frequencies were determined in two TK6-related human B lymphoblast cell lines that vary in p53 expression. TK6 cells express wild-type p53, WTK1 cells over-express a mutant form of p53, and NH32 cells express no p53 protein (8;37). Frequencies were determined in multiple (20-50) clonal isolates for each cell line. Alterations in p53 were associated with >10 fold higher spontaneous levels of chromosome instability. The average frequency of dicentrics/cell in unirradiated control TK6 clones was 0.002 ± 0.001, while that for NH32 was 0.041 ± 0.009, and 0.034 ± 0.007 for WTK1 cells. The effect of ionizing radiation on chromosome instability was also p53 dependent. There was little or no significant increase in chromosome instability levels after gamma irradiation in either NH32 or WTK1. In fact, there was suggestion of a slight decrease in dicentric frequencies in NH32 cells exposed to 10 cGy γ-rays. The maximum levels of chromosome instability observed appeared cell-line-specific. The maximum levels of dicentrics/cell are 0.02 dicentrics/cell for TK6, and between 0.033 and 0.041 dicentrics/cells for the genetically modified cell lines (NH32, WTK1). The results suggest that while radiation may be acting through effects on p53-dependent apoptotic processes, either the effect is not a direct effect on p53 gene expression or apoptosis function, or that the process by which radiation changes dicentric frequencies does not totally inactivate p53 function or apoptotic response.

Telomere Involvement: Dicentric chromosomes formed by end-to-end fusions at telomeres are often seen in tumor cells (1;3;22;24;39) and are sometimes found in cells from patients with specific genetic syndromes (11;21;38), from cells that have been altered genetically by viral infection or transfection (10;16;25;29;30), and in cells exposed to IR (27;29). Chromosome fusions result from loss of, or alteration in, telomere structure or function (2;7;12;14;35). Mammalian telomeres consist of TTAGGG repetitive sequences that end in a 3' single-stranded overhang. The overhang is able to fold back and invade the double-stranded region of the telomere forming a loop that is stabilized by a set of specialized proteins (12). These loops mediate capping the end of chromosomes by masking telomere ends from recognition as DSBs by DNA repair systems. Loss of capping can result in chromosome fusions, cell cycle arrest, and apoptosis (9).

There appear to be two general pathways by which telomeres can lose their structure and become susceptible to fusion. One is loss of sequence or telomere shortening. Telomere replication requires the specialized ribonucleoprotein enzyme, telomere terminal transferase (telomerase) to add telomere sequences and maintain telomere length (4;5) In the absence of telomerase activity, telomeres shorten progressively until normal telomere structure is lost (~6 kb (15)). At this point they become susceptible to chromosome fusions (9). Reintroduction of telomerase into the mouse telomerase deficient model or into

normal diploid fibroblasts that normally do not express telomerase activity reverses or prevents the phenotype of chromosome fusions. Almost all immortal cell lines contain active telomerase activity (13), yet they also show chromosome instability that is sometimes associated with telomere shortening (32;33). Thus there must be factors in addition to telomerase that affect telomere length dynamics and chromosome instability. The number of potential modifiers of telomere length is large. Some act by regulating telomerase activity itself or access of telomerase to telomeres while others act by altering the processing of telomeres post-replication (9). Some of the effects are indirect. For example, a number of studies have shown accumulation of breaks at telomeres after oxidative stress (6;23). There are also reports of defective repair of DNA damage at telomeres (17;18). Therefore, DNA breaks induced in telomeres might lead to rapid shortening. Alterations in telomere structure that lead to telomere fusions can also occur independent of telomere shortening. Telomeres form a complex with a large number of specific binding proteins that help maintain their protective structure. These proteins include TRF2, DNA PK (Ku70/86 and DNA-PKcs), and MRE11/RAD50/NBS1 that are required for post-replicative processing of telomere ends. Mutations in some of these elements lead to chromosome fusions in the absence of telomere shortening (2).

The involvement of telomeres in the chromosome fusions led us to investigate telomere lengths in all three cell lines and in clones isolated from each cell line. As compared to TK6, on average, telomeres were shorter in the p53-deficient WTK1 and NH32 cells. The range of telomere lengths were also greater. Telomere lengths ranged from 3.2 kb to greater than 25 kb in TK6 and clones derived from TK6. In WTK1, telomere lengths ranged from 1.3 to 21 kb, while in NH32, they ranged from 0.9 to greater than 25 kb. All three cell lines have telomeres that are short enough to lose structure and fuse with other short telomeres. There were no consistent significant variations in telomerase activity (30). All cell lines had approximately the same levels of telomerase activity, an observation that suggests that differences in telomere lengths are not due to differences in telomerase activity.

There were clonal variations in telomere lengths, and telomere lengths changed with time in the individual clones. For example, when telomere lengths in W1-25, a clone isolated from WTK1 cells, were first analyzed, the telomere lengths ranged from 1.0 to 2.7 kb. After culturing W1-25 an additional 20-30 generations, the range of telomere lengths increased to from 1.8 to 5.2 kb. Similarly, telomere lengths in subclones isolated from W1-25 showed wide variation in lengths, ranging from 1.1 to 13.9 kb. For this WTK1 clone, in general the subclones with the shortest telomeres were the most chromosomally unstable (30).

The dicentrics were analyzed to determine whether any telomere sequences could be observed at the sites of fusions. While most of the fusions (>75%) contained no cytogenetically identifiable telomere signal we did observe significant numbers of dicentrics with a cytogenetically observable telomere sequence at the site of fusion (29). As many of the normal TK6 chromosomes

lack an observable telomere signal, we cannot conclude that most dicentrics result from telomere sequence loss. The cytogenetic analysis is limited in its ability to detect small telomere sequences, but we can conclude that telomere loss does not underlie all of the chromosome fusions.

IR exposure had no apparent effect on telomere lengths in any of the TK6-derived cell lines. While originally we proposed that the fusions developed from telomere shortening (30), this hypothesis was primarily based on the W1-25 clone mentioned above. In this clone, subclones that showed high levels of instability also had short telomeres, and with time, as dicentric frequencies declined, telomere lengths appeared to lengthen. Our most recent studies suggest that the association between dicentrics and short telomeres might be indirect. First, there was no obvious relationship between telomere length and dicentric chromosome frequency when all three cell lines were considered together. In addition, while radiation exposure increased the levels of instability in TK6 and related cells, it had no measurable effect on telomere lengths. Thus the underlying defect in TK6 cells that leads to dicentric formation may not be short telomeres, but some alteration in telomere structure that leads to chromosome fusions (2;12;15;35). The p53-dependence on short telomere size, i.e., that the short telomeres were observed in the p53-deficient cells, might reflect some p53-dependent effect on recombination. It has been reported that p53 normally suppresses homologous recombination (20) and that the p53-deficient sister clones of TK6 show evidence of hyper-recombinogenic activity (36). Electron microscope studies have demonstrated the presence of p53 at t-loop junctions (34) and recently p53 has been shown to be associated with the telomeric complex (26).

Studies at the gene mutation level

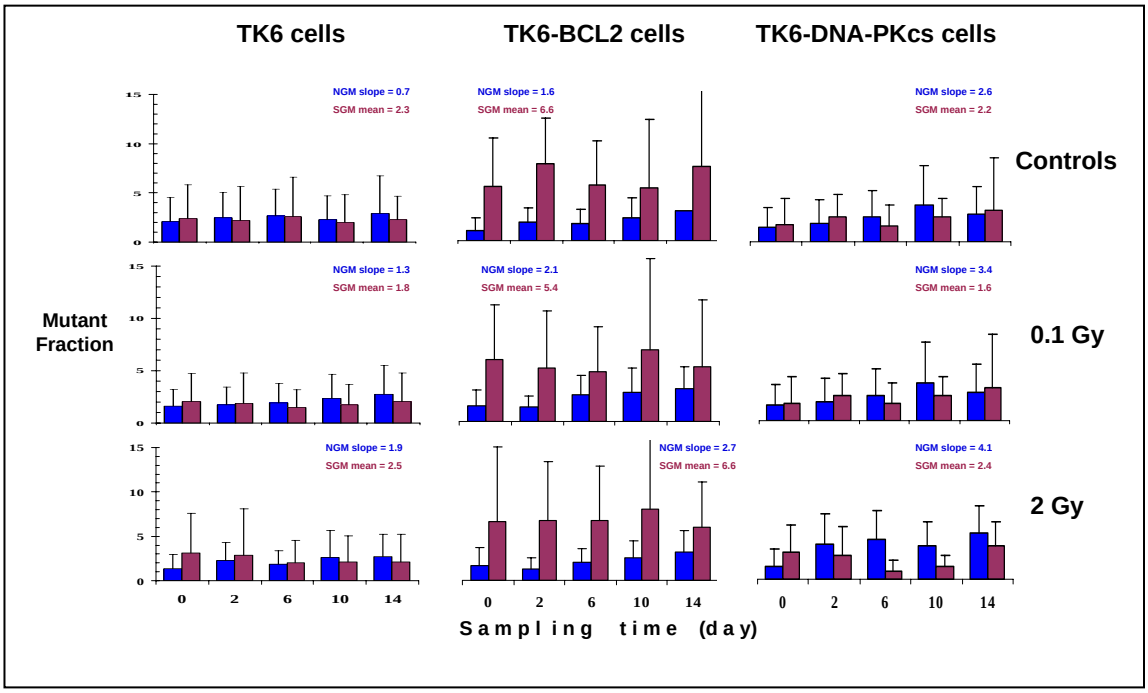
We measured mutagenesis at the thymidine kinase (tk) locus. We quantified mutant frequencies (MF) of normal growth mutants (NGM) in 44 independent clones at $2.73 \pm 0.78 \times 10^{-6}$/cells while for slow growth mutants (SGM) it was $2.39 \pm 0.52 \times 10^{-6}$/cells. These are similar to the frequencies observed for hprt mutants in human peripheral blood lymphocytes.

We then went on to compare MFs in cells that had survived low dose (0.1 Gy) or high dose (2 Gy) gamma-irradiation. These analyses were done 30-35 generations after the treatment, and for each case, up to 50 independent surviving clones were analyzed. For each clone, the MF was determined on 5 separate days over a period of two weeks. For NGM, which accumulate over time, mean MF values for each day were used to calculate a slope (i.e., a mutation rate), and these slopes were then used to compare the various conditions. For SGM, which reach a steady-state, the five determinations were used to generate a mean that was then compared among the different conditions

Furthermore, we varied the genetic status of the cells, so that at the time of irradiation cells were either (1) over-expressing bcl-2 and thus less able to

undergo radiation-induced apoptosis, or (2) had been knocked down for the non-homologous end joining repair protein DNA-PKcs, and thus were less able to repair double strand breaks by NHEJ.

The data for this set of experiments are shown in Figure XX



As can be seen in the leftmost panels of Figure XX, irradiation alone led to an increase in mutation rates of NGM, but had no effect on the generation of SGM. Over-expression of bcl-2 led to increases in mutation rate, but this was not radiation-dependent.
Depletion of DNA-PKcs at the time of irradiation did increase subsequent genomic instability, when measured as the rate of accumulation of NGM. Again, SGM fractions were not affected by the RNAi treatment.

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