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Genome Dynamics and Transcriptional Deregulation in Aging

Rita Busuttil, Rumana Bahar, and Jan Vijg

Buck Institute for Age Research, 8001 Redwood Blvd, Novato, CA 94945

Abstract

Genome instability has been implicated as a major cause of both cancer and aging. Using a *lacZ*-plasmid transgenic mouse model we have shown that mutations accumulate with age in a tissue-specific manner. Genome rearrangements, including translocations and large deletions, are a major component of the mutation spectrum in some tissues at old age such as heart. Such large mutations were also induced by hydrogen peroxide (H_2O_2) in *lacZ*-plasmid mouse embryonic fibroblasts (MEFs) and demonstrated to be replication-independent. This was in contrast to UV-induced point mutations, which were much more abundant in proliferating than in quiescent MEFs. To test if large rearrangements could adversely affect patterns of gene expression we PCR-amplified global mRNA content of single MEFs treated with H_2O_2 . Such treatment resulted in a significant increase in cell-to-cell variation in gene expression, which was found to parallel the induction and persistence of genome rearrangement mutations at the *lacZ* reporter locus. Increased transcriptional noise was also found among single cardiomyocytes from old mice as compared to similar cells from young mice. While these results do not directly indicate a cause and effect relationship between genome rearrangement mutations and transcriptional deregulation, they do underscore the stochastic nature of genotoxic effects on cells and tissues and could provide a mechanism for age-related cellular degeneration in postmitotic tissue, such as heart or brain.

Keywords

Single cell; cell-to-cell variation; genome instability; somatic mutations; gene regulation

Introduction

Mutations, that is, irreversible changes in DNA sequence organization, are inextricably linked to the evolution of different life forms by providing the substrate of natural selection. However, too many mutations, without a mechanism to escape their adverse effects, can be harmful to a cell population. The effect of random mutations was illustrated by Elena and Lenski who used *E.coli* to show that increasing the number of random mutations resulted in a linear decline of average growth rate (Elena and Lenski, 1997). Of course, populations of *E.coli* cells are not directly comparable to mammalian tissues and organs and similar experiments with mammalian cells are difficult to carry out because of the larger size and more complicated organization of their genomes. Nevertheless, somatic cells of mammals are also vulnerable to deleterious mutations, the accumulation of which over time in various tissues and organs has been suggested as a general explanation of aging and cancer (Szilard, 1959, Curtis, 1963, Vijg, 2006). In evolutionary terms, there would be no selective advantage to maintain somatic

Address for correspondence Jan Vijg, Ph.D. Buck Institute for Age Research 8001 Redwood Blvd Novato, CA 94945 Phone: 415-493-3625 FAX: 415-493-3640 Email: jvijg@buckinstitute.org

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genomes for extended periods of time after the reproductive period. In order to test the hypothesis that mutation accumulation in the somatic genome adversely affects fitness of mammals it is necessary to first quantify and characterize mutations in different organs and tissues and then demonstrate how mutation accumulation can exert adverse effects.

Measuring tissue-specific mutations in the mouse

Monitoring the accumulation of mutations in cell populations, especially in different tissues and organs *in vivo*, is not trivial. Curtis and co-workers were among the first to demonstrate an increase in chromosomal mutations with aging in mouse liver, using hepatocyte metaphases after partial hepatectomy (Curtis and Crowley, 1963). Later, both cytogenetic tests and the HPRT assay have indicated the accumulation of mutations with age in lymphocytes from humans and mice (Dempsey et al., 1993, Jones et al., 1995, Ramsey et al., 1995, Tucker et al., 1999). However, given that the large majority of cells in adult humans and animals rarely undergo cell division, such assays are a poor reflection of the *in vivo* situation. In order to extend these studies to organs and tissues of aging animals, we developed transgenic mouse models harboring chromosomally integrated bacterial mutation reporter genes, which can be recovered from their integrated state, transferred to *E.coli*, and then analyzed for mutations (Gossen and Vijg, 1993). One of these models uses a *lacZ* reporter as part of a plasmid, which can be excised and transferred into *E. coli* (Boerrigter et al., 1995). Using a positive selection system only those *E.coli* cells harboring a mutant *lacZ* gene can give rise to a colony (Fig. 1). Using this model, which is sensitive to a broad range of mutational events including point mutations, small insertions and deletions as well as large rearrangements with one breakpoint in a *lacZ* reporter and the other elsewhere in the mouse genome, we previously demonstrated that mutations accumulate in most tissues of the mouse during aging (Dollé et al., 1997, Dollé et al., 2000). Mutant frequencies at the *lacZ* transgene were found to increase with age in the liver, from about 4×10^{-5} in young adults to about 15×10^{-5} in 30-month old animals. An age-related increase was also found in the small intestine, spleen and heart, whilst there were only very small increases in mutant frequency in the brain and testes (Vijg and Dollé, 2002).

We also observed striking organ specificity with respect to the mutational spectra of the old animals. Interestingly, while a highly proliferative tissue such as the small intestine accumulated mainly point mutations, in organs such as liver and heart large genome rearrangement mutations were a prominent part of the spectrum (Vijg and Dollé, 2002). This suggests that postmitotic cells, such as the cardiomyocytes of the heart, can acquire mutations, independent of replication errors.

Are mutations replication-dependent?

To investigate whether mutations can be induced in non-replicating cell populations, we treated mitotically active or quiescent embryonic fibroblasts (MEFs) derived from the *lacZ*-plasmid transgenic mice with two different DNA damaging agents, 2.5 J/m² ultraviolet light (UV) or 100 μ M hydrogen peroxide (H₂O₂) and then analyzed the mutant frequencies at the *lacZ* locus. Cell survival at these doses is more than 80% (not shown). The proliferating cell population was allowed to divide in an unlimited manner by providing the cells with medium containing serum. Quiescent cells were prepared by starving the cells of serum, which was present in only 0.5% rather than the usual 10%. The cells were maintained under these conditions for the duration of the experiment and quiescence was confirmed by the very low levels of ³H-thymidine incorporation (Busuttil et al., 2006). *LacZ* mutant frequency determinations in these cell populations indicated that while UV-induced mutagenesis is highly dependent on cell proliferation, H₂O₂ treatment resulted in slightly more mutations in the quiescent cells than in the actively proliferating ones (submitted for publication) (Fig. 2). Interestingly, while the replication-dependent, UV-induced mutations were mainly point mutations, H₂O₂ treatment

resulted almost exclusively in the kind of genome rearrangement mutations observed to increase with age in the heart and liver of these same *lacZ*-plasmid mice (described above). Hence, these results suggest that point mutations are highly dependent on replication errors, while genome rearrangements might be a result of mistakes during annealing of double-strand breaks known to be induced by H_2O_2 . This latter type of event apparently does not require DNA replication for its fixation.

Interestingly, the observed replication-independent nature of genome rearrangements is in keeping with the very similar frequency of such mutations in the male and female germ line as a cause of heritable disease (Drake et al., 1998). This is in contrast to disease-causing point mutations, which occur much more frequently in males than in females, with the highest incidence occurring in older males (Crow, 2000). Since sperm are continuously produced throughout reproductive life, the number of cell divisions that have occurred increases with age and hence so does the total number of mutations. The ovum undergoes only 22 cell divisions prior to meiosis and two during meiosis with all cell divisions being completed before birth, explaining the lack of increase in point mutations with age in females. These findings are therefore in complete agreement with our results of point mutations being highly dependent on cell proliferation whilst rearrangements can occur in both dividing and non-dividing cell populations.

How do mutations exert adverse effects during aging?

It is easy to see how mutation accumulation can causally contribute to the well-documented exponentially increasing cancer risk during aging. Indeed, we have recently demonstrated a correlation between increased liver cancer in SOD1 null mice and the accumulation in the liver of GC to AT and GC to TA basepair substitutions, both signature mutations of oxidative stress (Busuttil et al., 2005). While such aging-related changes affecting apoptosis, cellular senescence, the immune response and probably a host of other systems are certainly contributing to the increased cancer risk at old age, there is little dispute regarding the role of random mutagenesis in cancer initiation and progression (DePinho, 2000). However, can randomly induced genome rearrangements also lead to non-cancer, degenerative symptoms of aging, more or less similar to the reduced growth rate of the *E.coli* cell populations mentioned at the beginning of this paper? More specifically, can random genome alterations lead to functional decline of such major postmitotic organs such as the heart or brain? One possibility is that random mutations deregulate normal patterns of gene expression and they are more likely to do so when they involve large genome rearrangements rather than point mutations (Vijg and Dollé, 2002). Highly differentiated, specialized cells exert their function through the activity of a limited number of interconnected functional pathways. Such networks are robust and not easily deregulated. But a gradual accumulation of large genomic mutations would likely have adverse effects after extended periods of time. Large deletions, for example, could lead to haploinsufficiency of a number of genes, including genes encoding transcription factors. This would have a ripple effect since it is known that the target genes of many transcription factors are sensitive to loss of heterozygosity. Translocations or inversions are likely to alter the relative position of gene regulatory regions relative to each other and their main transcription unit. Hence, a process of gradual genome alterations would increase gene transcriptional noise, thereby altering the effectiveness of the cell's network of functional pathways.

We decided to directly test for increased transcriptional noise in a cell population by quantitative analysis of mRNA levels of arbitrarily selected genes in single cells. For this purpose, a quantitative, unbiased procedure to amplify global mRNA from single cells is essential. We adopted the PCR-based method reported by Klein et al. (Klein et al., 2002), using a single poly-dC primer, making all amplified sequences equally GC-rich and allowing a high

annealing temperature. We then treated a population of proliferating, early passage *lacZ* MEFs with the same 100 μ M H₂O₂ as before. Individual cells were collected at 6 and 48 h as well as at 9 days after treatment and subjected to global mRNA amplification. The mRNA levels of *Actb*, *B2M* and *Tuba6* (all housekeeping genes) were determined by real-time PCR, using *Gapd* as the reference gene. As shown in Fig. 3A for *Actb*, untreated cells already display a trend towards variation in gene activity as compared to single cell equivalents (equivalents of single cells as pipetted from a pool of lysed cells). However, a dramatic increase in cell-to-cell variation of gene expression was observed 48 hours after treatment of the cells with H₂O₂. The time course shown in Fig. 3B indicates that cell-to-cell variation in relative gene expression at 6 h after treatment was not yet elevated. This suggests that H₂O₂-induced transcriptional noise is not due to direct chemical damage to the cells (to DNA, proteins or lipids), the effects of which would be expected to manifest immediately. The results also indicate that the increased stochasticity at 48 h is persistent because it was still present at 9 days after treatment (Fig. 3B). Most of the cells at this time exhibited a senescent phenotype, a known consequence of treatment with H₂O₂ (Parrinello et al., 2003).

We then determined the *lacZ* mutation load in these cells. Whilst some mutations were apparent as early as 6 h following exposure to H₂O₂, their frequency was substantially increased at 48h post treatment and somewhat reduced after 9 days (Fig. 3C). Molecular characterization of the mutants confirmed that most of these mutations were large rearrangements. The similarity in kinetics of the increase in transcriptional noise and H₂O₂-induced genome rearrangements makes it tempting to speculate that they are mechanistically related. However, at this stage no such cause and effect relationship has been established. Interestingly, we also compared the level of transcriptional noise in cardiomyocytes dissociated from fresh heart samples of young (6-month) and old (27-month) mice. Global mRNA amplification was followed by real-time PCR quantitation of mRNA levels of the same housekeeping as well as several heart-specific genes, using different reference genes. While gene expression levels already varied significantly among cardiomyocytes from young heart (as compared to single cell equivalents; not shown), this heterogeneity was significantly elevated at old age. This increased cell-to-cell variation was observed irrespective of the reference gene used (Bahar et al., 2006). An example of the increased cell-to-cell variation in transcript levels in old mouse heart is shown for β -actin (*Actb*), β -2 microglobulin (*B2m*), lipoprotein lipase (*Lpl*) and cardiac α -actin (*Actc1*), normalized over glyceraldehydes-3-phosphate dehydrogenase (*Gapd*) (Fig. 4). Since especially in heart genome rearrangement mutations were found to increase with age (Dollé et al., 2000), this result is not in contrast with the possibility that these mutations effectively increase transcriptional noise during aging, at least in the heart.

Discussion and future prospects

Mutation as a fundamental process is the driving force behind the evolution of genomes by virtue of the power of natural selection to guide the process over the heights of the fitness landscape rather than allowing it to result in population extinction. Such selection rarely applies to the somatic cells of multicellular organisms, possibly with the exception of certain populations of stem cells. Apart from cancer, a clonal disease, not many disease endpoints have been considered as the possible outcome of a process of mutation accumulation in somatic cells. While implicated as a general cause of aging, no specific mechanism has been proposed as to how mutation accumulation could ever lead to the multitude of degenerative processes that comprise aging. We have now demonstrated that a large variety of mutations accumulate with age at greatly different rates in a tissue-specific manner. More recently we have shown that while some organs, such as brain, do not seem to accumulate mutations with age at all, certain sub-structures are much more susceptible to mutagenesis and do show increased mutational loads at old age (unpublished results).

The observed increased levels of genome rearrangements, a highly toxic type of mutations, accompanied by increased cell-to-cell variation in gene expression, suggest that genotoxic stress may at least in part exert its adverse effects on organism by deregulating normal patterns of gene expression. Transcriptional noise has been described previously in *E.coli* and yeast. It is inherent in the basic process of transcription especially for genes with low expression levels (Beckes et al., 2005, Kaern et al., 2005) and increased noise in gene expression has been implicated in reduced organismal fitness (Fraser et al., 2004). It is possible that alterations in the somatic genome, for example, as induced by reactive oxygen species - a likely causal factor in aging - contribute to the observed increased stochasticity of gene expression.

Our present results underscore the importance of chance in aging and suggest a possible mechanism to explain how a stochastic process such as mutation accumulation can have functional consequences for cell populations organized in tissues and organs. It would be important to test other cell types that are subject to aging-related degeneration, especially the neuronal cells of the brain, for increased noise in the expression level of their genes. Indeed, although we detected only few genome rearrangements with age in brain, others observed an age-related increase in the loss of heterozygosity events (Bailey et al., 2004), a type of mutation that cannot be detected by using the lacZ system. Also aneuploidy in aging mouse neuronal cells, another type of event we cannot detect appears to be widespread in the brain (Rehen et al., 2005). Also chromatin alterations due to changes in DNA methylation or alteration of the histone code could greatly increase transcriptional noise in postmitotic cells. A more detailed examination of the possible mechanisms by which different postmitotic cell types can generate increased transcriptional noise through random genome alterations would open up new vistas on the role of genotoxic stress in aging and disease. In this respect, the role of mouse models harboring defects in genome maintenance, such as the repair of double strand breaks, is likely to become critically important (Hasty et al., 2003).

Abbreviations

MEF, mouse embryonic fibroblast; H₂O₂, hydrogen peroxide; UV, ultraviolet light; FBS, fetal bovine serum; DMEM, Dulbecco's Modified Eagle's Medium; MF, mutant frequency; Actb, β -actin; B2m, β -2 microglobulin; Lpl, lipoprotein lipase; Actc1, cardiac α -actin.

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Fig 1.

Schematic depiction of the *LacZ*-plasmid model for mutation analysis. In this system, plasmids are rescued by the excision of genomic DNA with *Hind*III, followed by their separation from the mouse genomic DNA using magnetic beads, precoated with a *lacI* repressor protein. The plasmids are then ligated and transferred to *Escherichia coli* C ($\Delta lacZ, galE^-$) using electrotransformation. A small amount of transformants is plated in medium with X-gal to determine the total number of plasmids rescued. The remainder is plated on the lactose analogue p-gal, to select only the cells harboring a mutant *lacZ* gene. The mutant frequency is the ratio of the colonies on the selective plate versus the colonies on the titer plate (times the dilution factor).

Fig 2. Frequencies of point mutations (hatched) and genome rearrangements (solid) in lacZ-MEFs, either irradiated with 2.5 J/m² UVC radiation or treated with 100 μ M H₂O₂ at 3-days post treatment.

Fig 3.

H₂O₂ treatment of MEFs increases cell-to-cell variation in gene expression. (A) Cell-to-cell variation in relative expression of *Actb* (normalized over *Gapdh*) among MEF single-cell equivalents, untreated MEFs and MEFs at 48 h after treatment with 0.1 mM H₂O₂. The variation among the treated cells is significantly greater than among the untreated cells ($P < 0.0001$). Boxes in the box plots indicate the interquartile range (IQR) with the median; the whiskers indicate 1.5x the IQR. (B) Coefficient of variance (CV) calculated for all relative quantities of *Actb*, *B2m* and *Tuba6* at each time point and treatment condition. The 6 h, 48 h and 9 day time points are based on 2, 4, and 3 independent experiments, respectively. Each experiment consists of 11-15 single cell determinations for each gene under each treatment condition. (C) *lacZ* mutation frequencies at 6 h, 48 h and 9 days after H₂O₂ treatment as compared with control populations. Mutation frequencies are averages from three independent determinations from each of two parallel experiments. The subdivision into point mutations (grey bars) and genome rearrangements (black bars) was made on the basis of restriction enzyme analysis of 48 mutants taken from each experiment. Error bars indicate s.d.

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Fig 4.

Increased cell-to-cell variation in gene expression among cardiomyocytes from the heart of old as compared with young mice. Examples of four genes showing statistically significant cell-to-cell variability in expression (*Actb*, *B2m*, *Lpl* and *Actc1*; normalized over *Gapdh*) ($p < 0.0001$ for all genes). Boxes in the box plots indicate the interquartile range (IQR) with the median; the whiskers indicate 1.5x the IQR.

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