Health Span and Life Span in Transgenic Mice with Modulated DNA Repair

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ABSTRACT: One way to better understand the contribution of DNA repair, DNA damage, and mutagenesis in aging would be to enhance DNA repair activity, lower DNA damage, and lower mutagenesis. Because the repair protein O^6-methylguanine–DNA methyltransferase (MGMT) acts alone and stoichiometrically, the human MGMT (hMGMT) cDNA was selected to test the feasibility of enhancing DNA repair activity in transgenic mice. MGMT activity is largely responsible for ameliorating the deleterious effects of O^6-methylguanine (O^6mG) lesions in DNA in a direct reversal mechanism. A transgene was constructed consisting of a portion of the human transferrin (TF) promoter and hMGMT cDNA such that hMGMT is expressed in transgenic mouse brain and liver. Expression of hMGMT was associated with a significant reduction in the occurrence of an age-related hepatocellular carcinoma in male mice at 15 months of age. Longitudinal and cross-sectional studies were initiated to determine whether the reduced incidence of hepatocellular carcinoma would impact median or maximum life span. The cross-sectional study performed on 15-month-old male animals confirmed the reduced occurrence of spontaneous hepatocellular carcinoma. At 30 months of age, however, the occurrence of hepatocellular carcinoma in at least one transgenic line was similar to that for nontransgenic animals. The longitudinal study is ongoing; however, at present no significant differences in life span have been detected. Tissues expressing the MGMT transgene also displayed greater resistance to alkylation-induced tumor formation. These results suggest that transgenes can be used to direct enhanced DNA repair gene expression and that enhanced expression can protect animals from certain spontaneous and induced tumors.

KEYWORDS: Transgenic mice; Alkylation damage; Aging; Hepatocellular carcinoma; MGMT

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INTRODUCTION

An age-related decline in genomic integrity has been proposed to be a fundamental mechanism in the aging process.[1–4] The somatic mutation theory of aging encompasses the tenant that accumulation of mutations with time results in the inactivation of genes, decreased protein function, and ultimately cell death. The DNA damage theory of aging embraces the concept that accumulated DNA damage increasingly interferes with DNA replication and transcription, which in turn impairs the ability of cells to perform their functions, thereby leading to aging. More recently it has become accepted that only a certain amount of DNA damage is tolerated before cells commit themselves to a pathway of cell death. These two theories of aging, which are based on genomic integrity, are not mutually exclusive and are difficult to study independently of each other. Some of the data that support the role of genetic integrity in aging are discussed briefly below.

Genetic integrity at the level of the chromosome was among the earliest observations demonstrating a decreased integrity with increased age. For example, chromosomal aberrations have been shown to increase in preparations from mouse liver[5] and mouse kidney cells[6] with older age. The majority of studies involving humans have been performed with peripheral blood cells and have also revealed increases in chromosomal aberrations with increased donor age.[7–13] Thus, there are data linking increased age with increased chromosome aberrations.

Genetic integrity has also been assessed at the level of individual genes, and increases in mutation frequencies have been observed with increased age. The *Hprt* gene can be scored for mutations using selective media and is commonly used for assessing mutation frequencies in humans and other mammals. Several studies have reported an increase in mutation frequency in the *Hprt* gene with increased age in various cell types, but principally in peripheral blood lymphocytes.[12,14–18]

Genomic integrity has been shown to be compromised in male and female germ cells as well. For example, in humans there are clear correlations between increased maternal age and pregnancies with chromosomal abnormalities.[19] In addition, some autosomal dominant disorders display a higher *de novo* germline mutation frequency with increased paternal age. New mutations giving rise to achondroplasia, Marfan's syndrome, and Apert's syndrome are associated with increased paternal age.[20]

More recently, transgenic mouse models have been developed that facilitate assessing mutation frequency for the transgene in any tissue and at any age. Two transgenic models have been developed that utilize shuttle vectors so that individual copies of the transgene can be isolated after being in the mouse and grown as single colonies in appropriate *E. coli* strains. Mutant transgenes are identified by plating the *E. coli* onto agarose with a chromogenic substrate or a selective agent. Both models use a component of the bacterial *lac* operon. Important information about mutation frequency and mutation spectra are being generated with these models. It has been shown in the *lacI* mouse model that the mutation frequency increases approximately fourfold in DNA obtained from spleen as the animals age from birth to 24 months.[21] Similarly, the mutation frequency increases for the *lacZ* transgene in DNA obtained from liver.[22] Nevertheless, the mutation frequency was not observed to increase with age for the *lacZ* gene in DNA obtained from brain.[22] A paternal age effect on mutation frequency in the *lacI* gene has been observed such that a 10-fold increase was

noted between 60-day-old mice and 28-month-old mice.[23] Although the data from transgenic mice largely support the theory of decreased genomic integrity with aging, it also appears that changes in genomic integrity may not occur in all tissues as an animal ages.

Dietary restriction is the one experimental intervention known to increase maximal life span in several species.[24] The increase in longevity is associated with a decrease in oxidative DNA damage[25,26] and mutations in the *Hprt* gene.[18] The association between extended life span and reduced DNA damage and mutation supports the somatic mutation and DNA damage theories of aging.

Data also exist that do not support the contribution of decreased genetic integrity to aging. For example, a recent analysis of the effect of dietary restriction on mutation frequency in the *lacI* transgenic mouse model found no significant difference between *ad libitum* and dietary-restricted animals at 6 and 12 months of age.[27] Notably, a 12-month-old mouse has not even begun to approach the median life span. Thus, it is not clear that a conclusion can be made about the effect of dietary restriction on the accumulation of mutations over a lifetime. Furthermore, most DNA repair deficiency syndromes are not associated with premature aging, with the exception of an increased susceptibility to carcinogenesis.

ASSESSING THE IMPACT OF ELEVATED DNA REPAIR ON AGING

The data concerning genetic integrity with old age are largely correlative. This, in combination with the variable results regarding genetic integrity with old age, has contributed to vacillations in enthusiasm for the theories involving genetic integrity in aging. One way to assess the contribution of genetic integrity to aging more directly would be to alter the levels of spontaneous DNA damage and mutation and determine the resulting impact on aging. The levels of DNA damage and mutation are in part a reflection of DNA repair activity. Thus, one potential way of altering spontaneous DNA damage and mutation levels would be to alter DNA repair activity. DNA repair could be reduced or enhanced to achieve altered levels of damage and mutation. We have chosen to try to enhance DNA repair because reductions in DNA repair seem more likely to result in substantial increases in pathology and anomalies that would render the assessment of aging more difficult. Furthermore, enhanced DNA repair might mimic the reduction in DNA damage and mutation observed in conjunction with dietary restriction and have an impact on life span, one quantitative measure of aging.

Most DNA repair mechanisms involve multiple proteins, and it is not presently known which steps are limiting in the pathways. Nevertheless, a few repair processes are performed by single proteins and as such would be more readily amenable to manipulation in a transgenic mouse system. O^6-methylguanine–DNA methyltransferase (MGMT) is a DNA repair protein that removes alkyl lesions at the O^6 position of guanine. It was selected to test the feasibility of overexpressing a DNA repair protein that would then have a biological impact using transgenic mouse technology. MGMT was chosen because (1) it acts in solo, (2) it directly reverses O^6-alkylguanine DNA lesions, (3) it acts stoichiometrically, (4) its activity has been well characterized in mammals, and (5) the lesion it repairs is highly mutagenic and carcinogenic.

The transgene consists of a portion of the human transferrin (TF) promoter, which has been shown to direct robust expression of various genes in brain and liver of transgenic mice.[28–30] The hMGMT cDNA was placed downstream of the promoter and was followed by an SV40 polyadenylation signal.[30] Transgenic mice were generated in two different strains of mice, C57BL/6J and C3HeB/FeJ.[30] The hMGMT transgene was shown to be expressed in brain and liver as expected from previous studies.[30,31] One line in the C3HeB/FeJ strain, however, fortuitously expressed the transgene in lung, and the transcript was slightly larger than expected based on the construction of the transgene[31] and from the transcript size in the seven other lines for which expression was assessed.

Immunohistochemistry was performed with monoclonal antibodies specific for hMGMT to determine which cell types in the various tissues were actually expressing the transgene. The hMGMT protein was detected in hepatocytes in liver, cells that are probably glial cells in brain, and cells in lung that are likely pulmonary interstitial macrophages. The unexpected expression in lung was probably the result of the transgene integrating into a locus that is normally expressed in pulmonary interstitial macrophages. In all cases, the hMGMT protein was found principally in the nucleus. Western blot analysis revealed the protein was the appropriate size in all expressing tissues.[30]

Because the spontaneous level of O^6-methylguanine (O^6mG) is so low it would be difficult to quantitatively measure a decrease of the lesion in DNA, we performed *in vitro* DNA repair assays using a substrate that is recognized and repaired by MGMT. A significant increase in MGMT activity was observed for tissues expressing the transgene.[30,31] Next, our transgenic animals were crossed with *lacI* transgenic mice, and the mutation frequencies in TF/MGMT double transgenic mice compared to single *lacI* transgenic mice with DNA obtained from liver of young adult male animals. No significant differences were found in the spontaneous mutation frequencies;[31] however, this is probably due to the fact that spontaneous O^6mG lesions occur so infrequently that they do not contribute substantially to mutation frequencies.

Male TF/MGMT transgenic mice were then treated with a direct-acting alkylating agent to determine whether the carcinogenic effects of induced O^6mG lesions were reduced in tissues expressing the transgene compared to C3HeB/FeJ control mice. The alkylating agent selected was methylnitrosourea (MNU) because its tumorigenic activity is well established and because it generates O^6mG lesions. Mice were treated monthly with MNU by tail vein injection beginning at 3 months of age until 175 mg/kg body weight had been delivered through a total of seven injections. The animals were euthanized when moribund or at 15 months of age.

A summary of the nonhepatic tumors resulting from MNU treatment is shown in TABLE 1. A significant difference was observed in the prevalence of bronchioloalveolar carcinoma between the untreated control C3HeB/FeJ mice and LC22I animals treated with MNU compared to MNU-treated C3HeB/FeJ and LC26I mice. A difference approaching significance ($p = 0.058$) was detected for gastric squamous cell carcinoma in animals treated with MNU compared with untreated control mice. Increases were also observed for small intestine adenocarcinoma and lymphosarcoma, although these were not significantly different from untreated controls. These data demonstrate that the MNU delivered was an effective carcinogenic agent.

TABLE 1. Prevalence of various tumors among MNU-treated male mice and control male mice

	Mouse line			
Tumor	C3HeB/FeJ	C3HeB/FeJ + MNU	LC22I + MNU	LC26I + MNU
Gastric squamous cell carcinoma	0	41	29	30
Small intestine adenoma	10	24	19	17
Bronchioloalveolar carcinoma[a]	0	18	0	33
Lymphosarcoma	0	12	14	15
Total number of animals[b]	10	17	21	46

NOTE: Prevalence is expressed as the percent animals bearing the tumor.
[a]Denotes a tumor type for which a significant difference in prevalence was detected.
[b]Total number of animals equals tumor-bearing and tumor free.

TABLE 2. Prevalence of hepatocellular carcinoma in MNU-treated mice compared to untreated control mice

	C3HeB/FeJ	C3HeB/FeJ + MNU	LC22I + MNU	LC26I + MNU
Hepatocellular carcinoma	60	12	0	11
Total number of animals[a]	10	17	21	46

NOTE: Prevalence is expressed as percent of animals displaying the tumor.
[a]Total number of animals equals tumor-bearing plus tumor-free animals.

TABLE 3. Prevalence of spontaneous hepatocellular carcinoma

	C3HeB/FeJ	LC22I	LC26I	LC28I
Hepatocellular carcinoma	60	19	18	13
Total number of animals[a]	10	31	22	16

NOTE: Prevalence is expressed as the percent of animals bearing the tumor.
[a]Total number of animals equals tumor-bearing plus tumor-free animals.

One unexpected result was the reduction in hepatocellular carcinoma observed in male MNU-treated mice compared to control animals (TABLE 2). Without MNU treatment the prevalence of hepatocellular carcinoma was 60% in untreated C3HeB/FeJ male mice; however, C3HeB/FeJ, LC22I, and LC26I mice treated with MNU displayed a hepatocellular carcinoma prevalence of ≤12% ($p = 0.001$). Another surprising result was the observed reduction in spontaneous hepatocellular carcinoma in each of three independent transgenic lines (TABLE 3). Because MGMT activity is relatively high in liver in nontransgenic mice and the spontaneous occurrence of O^6mG is low, we expected that there was sufficient MGMT activity to counteract spontaneous O^6mG lesions in liver and did not consider that alkylation damage might contribute to spontaneous hepatocellular carcinoma. Indeed, the level of

TABLE 4. Prevalence of hepatocellular carcinoma in 15–16- and 28–31-month-old mice

Line	15–16 months old	Total No. animals[a]	28–31 months old	Total No. animals
Nontransgenic	50%	10	56%	16
LC22I	10%	10	58%	12
LC26I	30%	10	In progress	12

NOTE: Prevalence is expressed as the percent of tumor-bearing animals.
[a]Total number of animals is the number of tumor-bearing and tumor-free animals.

TABLE 5. Proportion of animals with probable cause of death due to hepatocellular carcinoma in longitudinal life span study

	Nontransgenic	LC22I	LC26I
Hepatocellular carcinoma	13 (35%)	16 (46%)	16 (52%)
Total number of animals	37	35	31

O^6mG in liver of C3HeB mice is not different from that in a nonsusceptible strain such as C57BL/6.[32,33] There are several possible explanations for the reduced spontaneous hepatocellular carcinoma in male C3HeB/FeJ mice. First, alkylation damage is not adequately repaired and does contribute to tumorigenesis in this strain. Second, accessibility to O^6mG lesions may be limited, and overexpression of MGMT corrects the limitation through mass action. Third, hMGMT may have a greater effective repair activity than murine MGMT. Fourth, MGMT may have as yet undetermined substrates or additional activities. Fifth, the TF promoter may compete for transcription factors, which in turn impacts the expression of genes involved in increased susceptibility to hepatocellular carcinoma. Sixth, the immune system may have been altered such that removal of transformed liver cells is improved. Regardless of the mechanism, a larger fraction of male animals remain free of hepatocellular carcinoma for an extended time when MGMT is overexpressed, thereby improving the health span of the animals.

The reduced occurrence of a normally high-frequency tumor presented the opportunity to test whether life span would be correspondingly affected. Accordingly, a longitudinal life span study was initiated, and an adjunct cross-sectional study was begun, both exclusively using male animals. The first cross-sectional study was performed on 15-month-old animals and supported the previous finding of reduced hepatocellular carcinoma at this age (TABLE 4). Notably, the prevalence of hepatocellular carcinoma was similar among transgenic and control mice approximately 30 months old (TABLE 4), thereby indicating that the protection from hepatocellular carcinoma afforded to the transgenic mice was not sustained. The longitudinal study is still in progress, but the current Kaplan-Meier estimate of the survival curve is shown in FIGURE 1. No significant differences in survival curves have been detected at this time. Furthermore, the preliminary results suggest that the prevalence of hepatocellular carcinoma is not reduced in the transgenic mice at the end of their natural lives (TABLE 5).

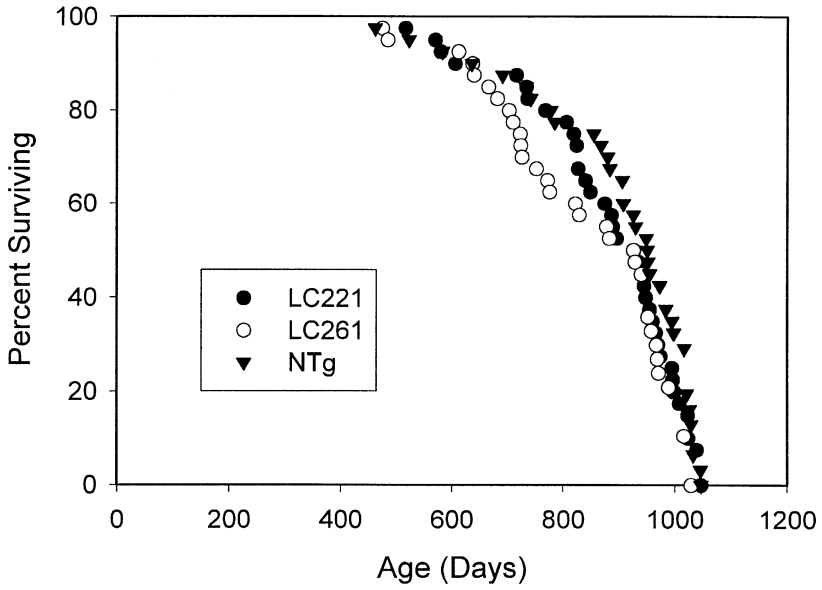

FIGURE 1. Survival curves for two transgenic C3HeB/FeJ transgenic lines and nontransgenic littermates. No significant differences have been observed between any of the mouse lines. LC22I and LC26I refer to two independent transgenic lines. NTg refers to nontransgenic littermates.

SUMMARY

Transgenic mice in the C3HeB inbred strain have been generated that have elevated expression of MGMT in brain and liver, and in lung of one line. The protein is of an appropriate size and is predominantly localized in nuclei of expressing cells. Expression of the transgene correlates with a reduced prevalence of MNU-induced tumors in liver in two independent lines and in lung of one line that fortuitously expresses in this tissue. Spontaneous hepatocellular carcinoma was reduced in three independent lines expressing the transgene in liver at 15 months of age; however, the reduced tumor prevalence was not observed at 30 months of age. Furthermore, a substantial number of transgenic and nontransgenic animals in the longitudinal study appear to die from the hepatocellular carcinoma. No significant impact on life span has been observed in conjunction with delayed onset of hepatocellular carcinoma.

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