The effect of dietary restriction during development in utero on the frequency of spontaneous somatic mutations

Lórien E.Newell and John A.Heddle

Department of Biology, York University, Toronto, M3J 1P3 Canada

Caloric or dietary restriction is known to be protective against cancer in humans and in mice but the mechanism is uncertain. Given that somatic mutations are important in carcinogenesis, dietary restriction may act by changing mutation rates. Indeed, previous studies have shown that reductions in caloric intake during development or in adult life make mice less susceptible to high doses of mutagens. In these studies there have been hints that the spontaneous mutant frequency may also be reduced, but no significant decrease has been observed save in one study of very old mice. Since the spontaneous mutant frequency is already low, reductions from this level require the use of much larger sample sizes than usual and larger than those used in the previous studies. As pre-existing mutations cannot be eliminated, it is necessary to reduce the dietary intake over a period of time when a substantial proportion of spontaneous mutations arise in order to see an effect. To overcome such problems, the dietary restriction in this study was applied during the time of the highest mutation rate, early development, and many more than the usual number of animals were studied. SWR female mice were crossed with Muta™Mouse males to obtain F1 progeny for analysis of mutant frequency. At conception, the dams were put into two groups, one that was fed ad libitum and another which was fed 80% of the ad libitum diet. Pups were killed at birth, DNA was extracted from the whole animal and used to measure the mutant frequencies of the mice at the cII locus. Although the weights of the pups from dams whose diet was restricted were significantly less than those of the ad libitum mice (P = 0.003), the litter sizes in the two groups were approximately the same and did not differ significantly (P = 0.13). There was no significant difference in the mutant frequencies in the dietarily restricted and ad libitum groups (P = 0.43). In addition, there was no significant correlation between the weights of the pups and their mutant frequency in either the ad libitum or dietarily restricted groups (r² = 0.14 and r² = 0.024). No difference was observed in mutant frequency between the ad libitum and dietarily restricted mice from litters of the same size (P = 0.61). These results indicate that the protective effect of dietary restriction on cancer rates is not mediated by an alteration in the spontaneous rate of mutation but rather by another mechanism, such as its effect on induced mutation.

Introduction

Spontaneous mutations have been implicated as a causative factor in aging, carcinogenesis and cell injury (see for example Loeb and Cheng, 1990). Somatic mutations are clearly involved in carcinogenesis, so reductions in their frequency would be beneficial. It has been shown that diet is an important factor in human cancers (Armstrong and Doll, 1975), thus it seems logical that alterations in diet can lead to the prevention of some types of cancer. It has been found that dietary restriction significantly reduces spontaneous and chemically induced tumor incidence in rodents (Albanes, 1987). In human studies it has been found that a restriction in caloric intake resulted in a reduction in rectal cell proliferation, which is a biomarker related to colon carcinogenesis (Steinbach et al., 1994). Studies have also shown that maternal diet may influence cancer incidence in offspring later in life (Strick et al., 2000; Hilakivi-Clarke et al., 2001). One method of determining the relationship between diet and mutation is through the use of transgenic rodents such as the Muta™Mouse system, as the mutant frequency can be examined in any tissue by recovering the λ vector from the DNA of that tissue (Gossen et al., 1989).

Previous studies have shown that a reduction in the diet of mice can lower the induced mutant frequency as compared with mice fed ad libitum (Casciano et al., 1996; Shima et al., 2000). However, the results of studies examining the spontaneous mutant frequency have not been as conclusive. Although Casciano et al. (1996) did not find a significant decrease in the spontaneous mutant frequency in lymphocytes of calorically restricted animals as compared with those in animals fed ad libitum, they did find lower spontaneous mutant frequencies in restricted mice. Similarly, Shima et al. (2000) observed that restriction of the caloric intake of dams before birth and during lactation and of their pups after weaning did not significantly reduce the spontaneous mutant frequency of the pups. In both studies the experiments were meant to examine the induced mutant frequency of mice and the spontaneous mutant frequencies were only meant as controls, thus the numbers of animals in each group were small (5–10). When investigating the spontaneous mutant frequency it is important to examine a large number of animals and to ensure that high enough titer are obtained to enable proper statistical analysis as the spontaneous mutant frequency is much lower than the induced mutant frequency. In one study of spontaneous mutation at the hprt locus, Dempsey et al. (1993) found a large increase in mutation in the oldest mice fed ad libitum, but this was absent from the calorically restricted mice, indicating protection against spontaneous mutation. Such large increases in mutation in old age have, however, not generally been found at transgenic loci in mice (Lee et al., 1994; Ono et al., 1995; Nishino et al., 1996), although genome rearrangements have been found in older mice (Dollé et al., 1997). DNA sequence analysis has revealed no significant changes in mutational specificity at the lacI transgene in any tissue at any age (Stuart et al., 2000a), and the mutation frequencies at the lacI transgene were shown not to be age dependent (Nishino et al., 1996).

1To whom correspondence should be addressed. Tel: +1 416 736 2100; Fax: +1 416 736 5698; Email: jheddle@yorku.ca

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Here we report the findings of an experiment that examined the effects of dietary restriction on spontaneous mutation frequencies in the F1 offspring of a SWR female×MutaMouse male cross in which the dams (SWR females) had their complete diet reduced by 20% from conception to birth. The mutant frequency was measured at the cII transgene, originally described in the Big Blue system (Jakubczak et al., 1996) and then adapted to the MutaMouse system (Swiger et al., 1999). The number of animals used in this study (n = 29 ad libitum; n = 27 dietarily restricted) were much greater than those used in previous studies examining the spontaneous mutant frequency (Dempsey et al., 1993; Casciano et al., 1996; Shima et al., 2000). Experiments of this type typically only investigate mutant frequencies of five or six animals per treatment group, whereas this study examines five times as many samples, thus providing a more reliable estimate of the effect of dietary restriction on the spontaneous mutant frequency.

Materials and methods

Feeding and care of SWR females

Females were fed AIN-93G (ICN) diet for 2 weeks prior to the start of breeding. During this acclimatization period the females were kept in cages of 10, subsequently being separated for mating. During mating both males and females were fed AIN-93G ad libitum. At conception dams were randomly assigned to two groups, an ad libitum group and a dietarily restricted group. The ad libitum group was fed AIN-93G ad libitum and the other was restricted to 80% of the amount the ad libitum group ate. Females were weighed every day to ensure that they were healthy and to diagnose pregnancy. On two occasions mice that were dietarily restricted were killed as their body weights decreased by >20% during pregnancy.

Detection of pregnancy

Vaginal smears were taken from each female in the morning to determine the stage of the estrous cycle. Approximately 40 µl of sterile water was inserted into and then removed from the vagina of the mice using a pipette. Slides were stained with Giemsa for identification. If females were in the diestrous, proestrous or early estrous stages, they were placed with MutaMouse males for breeding. Pregnancy was assumed if the female was in the appropriate stage and a vaginal plug was present. Once females were found to be pregnant, they were caged singly and were randomly assigned to either the ad libitum or dietarily restricted groups.

DNA extraction

At birth, pups were weighed and then killed by freezing in liquid nitrogen. They were then stored in a −80°C freezer until use. Frozen pups were ground up using a mortar and pestle and excess liquid nitrogen. An aliquot of 4 ml of lysis buffer with 20 mg/ml proteinase K and 10 µl/ml EDTA (TE) buffer at 55°C overnight. The tubes were subsequently incubated for 90 min at 30°C. Aliquots of 4 ml of a 1:1 phenol/chloroform mixture were added to each sample. The samples were left in a water bath at 55°C for 3 h. An aliquot of 4 ml of 1:1 phenol/chloroform mixture was added to each tube, which was then centrifuged in a benchtop centrifuge at 3500 r.p.m. for 10 min. The aqueous layer of the mixture in each of the samples was removed and put into a new test tube. To this was added 4 ml of the 1:1 phenol/chloroform mixture and the procedure was repeated once or twice more depending on the clarity of the aqueous layer. Chloroform (4 ml) was added to each test tube, the tubes were inverted and then centrifuged at 3500 r.p.m. for 10 min. The aqueous layer was again removed and then the DNA was precipitated out using 100% ethanol at a volume twice that of the aqueous layer.

Purification of DNA

DNA was dialysed by inserting the DNA sample with a pipette into dialysis tubing (10 mm diameter) and securing the ends with clips. The DNA samples were placed in a large flask with 4 L of sterile Tris–EDTA (TE) buffer at 4°C and stirred continuously. The DNA samples were allowed to remain in the buffer for 4 h at a time, after which time the solution was replaced with 4 L of fresh TE buffer twice.

cII assay

Stratagene’s Transpack reactions were used for the cII assay. Aliquots of 12 µl of dialysed DNA was added to each of the first reaction tubes (orange). The tubes were subsequently incubated for 90 min at 30°C. Aliquots of 12 µl from the second reaction tubes (blue) were added to each of the first (orange) tubes. The tubes were then incubated at 30°C for 90 min. The reactions were stopped by the addition of 500 µl of SM buffer and vortexing. To determine titres, 60 µl of a 1:100 dilution was added to 200 µl of G1250 and the mixture was allowed to sit at room temperature for 30 min to allow adsorption to occur. Titre plates were allowed to incubate at 37°C overnight. Mutants were measured by plating five tubes, each with 100 µl of the stopped reaction and 200 µl of bacteria, and were allowed to sit at room temperature for 30 min. These plates were allowed to incubate at 24°C for 2 days and then were counted to determine the mutant frequency.

Statistical analysis

t-Tests were performed on data that were normally distributed, as determined by the F-test, measuring homogeneity of variance. t-Tests were calculated using Microsoft Excel. If samples failed the F-test, the Mann–Whitney test was performed using Minitab®. Standard deviations used to calculate the F-test were found using Microsoft® Excel. Each mouse was used as an independent sample as in this study, as in previous studies (Passhius-Lew and Heddle, 1998), there was no detectable correlation in mutant frequencies between siblings.

Results

General observations

It was found that restriction of the diet of the SWR females after mating had an affect on the ability of the mice to become pregnant. The mice that were fed ad libitum were able to become pregnant 80% of the time, as compared to 44% in the dietarily restricted mice. Once the mice became pregnant, both groups were able to produce healthy litters. After the females gave birth, the weight of those in the dietarily restricted group (mean 20.3 ± 0.43 g) was significantly less than those in the ad libitum group (mean 21.8 ± 0.24 g, P = 0.004). The litter size of dietarily restricted dams (mean 7.7 ± 0.57 pups) was higher than the ad libitum group (mean 6.9 ± 0.44 pups), but not significantly (P = 0.13). Comparisons were made to examine the relationship between weight and litter size. In both the ad libitum and dietarily restricted groups there was a correlation between weight and litter size (r² = −0.34 and r² = −0.79, respectively) (Figure 1).
Dietary restriction and in utero mutations

Fig. 2. Comparison of mutant frequency in the ad libitum and dietarily restricted groups. The cII assay was performed to measure the mutant frequency of both groups. $n = 28$ in the ad libitum group and $n = 26$ in the dietarily restricted group, as the mutant frequency from one animal in each group was omitted given that fewer than 30,000 plaques were obtained. Error bars represent the standard error.

In both the ad libitum and dietarily restricted groups there were litters of eight pups. There was no difference between the mutant frequencies in the dietarily restricted and the ad libitum groups with litter size eight ($P = 0.61$) (Figure 4).

Discussion

The weights of the pups in the dietarily restricted group were significantly less than those in the ad libitum group, as was expected. This was due to less nutrients being available for development of the pups during gestation in the dietarily restricted group. Although the dams in the dietarily restricted group had more difficulty becoming pregnant, once pregnant the dams had normal healthy pregnancies in both groups and the litter sizes were approximately equal. Dams were restricted at conception rather than before to ensure that they would have a high enough proportion of body fat to be able to conceive. Previous studies have also shown that dietary restriction at the time of conception is sufficient to decrease the induced mutant frequency as compared with animals fed ad libitum (Casciano et al., 1996; Shima et al., 2000).

Previous studies have shown that there is no decrease in the spontaneous mutant frequency in mice (aged 6 and 12 months) that have been dietarily restricted (Stuart et al., 2000b). The animals in this experiment were killed at birth, as it has been found that there is a higher rate of accumulation of spontaneous mutations before birth (Passhius-Lew and Heddle, 1998). Although the mutant frequency is somewhat lower at birth than later, it is still easily measurable. DNA was extracted from the entire animal at birth as the animals were not of a sufficient size to allow for proper dissection of individual organs. Although previous experiments measuring spontaneous mutant frequencies have been performed on individual tissues, studies have shown that at birth the spontaneous mutant frequencies are similar in several tissues (Nishino et al., 1996; Ono et al., 2000).

It was expected that the mutant frequency of the mice in the dietarily restricted group would be less than that of the ad libitum group, as the weights in the restricted group were

Mutant frequency

When the cII assay was initially performed, the titres were insufficient (<20,000 p.f.u./reaction) for proper statistical analysis. Dialysis of the DNA resulted in acceptable titres (average titre ~130,000). There were only two instances in which samples were excluded due to low titres (see Figures 2 and 3). When the mutant frequencies of the two groups were compared, the spontaneous mutant frequency was greater in the ad libitum group, but not significantly so ($P = 0.43$) (Figure 2). The mutant frequencies were compared to the weights of the pups from both the dietarily restricted as well as the ad libitum groups at birth to examine if there was a correlation between mutant frequency and weight (Figure 3), but the results were shown not to be significant ($r^2 = 0.055$).

Fig. 3. The relationship between mutant frequency and weight. Two observations have been omitted given that fewer than 30,000 plaques were obtained, one ad libitum (MF = 4.14×10^{-4}) and one dietarily restricted (MF = 3.21×10^{-4}). ■, ad libitum animals; ○, dietarily restricted animals.

Fig. 4. A comparison of mutant frequencies of pups from ad libitum and dietarily restricted groups. Eight animals from each group were sampled. ■, ad libitum animals; ○, dietarily restricted animals; ×, the average mutant frequency of each group. Error bars represent standard error.
found to be significantly less. A higher weight suggests that there is more cellular proliferation, which implies that there have been more chances for replication errors or changes in the DNA to occur, thus resulting in a higher number of spontaneous mutations (Haas et al., 1993). On the other hand, in principle a single extra cell division in the history of each cell would be significant to double the weight of the animal, which would be more than the weight difference observed. Evidently, the difference in the number of cell divisions was insufficient to affect the spontaneous mutant frequency significantly.

Previous experiments examining the effect of dietary restriction on the induced mutant frequency have shown that there is a protective effect against carcinogen-induced mutation following a reduction in diet. The results obtained in this study failed to show a comparable effect on the spontaneous mutant frequency. This suggests that there is a different mechanism by which dietary restriction lowers the induced mutant frequency than the spontaneous frequency. Thus, the benefits of reducing caloric intake as a preventative measure against cancer is probably not due to a reduction in the spontaneous mutant frequency, but rather to a reduction in somatic mutations induced by environmental agents.

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