CHANGES IN STRUCTURAL INTEGRITY OF HEART DNA FROM AGING MICE

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Summary

The state of the structural integrity of the DNA from mouse myocardial cells has been investigated by utilizing both CaCl density gradient sedimentation and digestion by S1 endonuclease from Aspergillus oryzae. The DNA from myocardial cells of young mice sediments in a narrow peak at the expected density of 1.701 g/cm³, while the DNA from the heart cells of senescent mice became broadly distributed in CaCl gradients, banding even more multimodally in alkaline sucrose gradients. This mode of sedimentation indicates that old mouse DNA becomes partially fragmented. When the native DNA of myocardial cells from 6, 20 and 30 month old mice was treated with single-strand specific S1 endonuclease, it was the DNA from the senescent mice that showed a progressive increase in sensitivity to digestion by the enzyme. The results indicate that the heart DNA of aging mice develops single-stranded gaps in addition to a breakdown into differently sized fragments.

The survival of a given species is most successfully assured by the maintenance of the structural integrity of its genome. As a provision for this metabolic requirement, cells are equipped with enzymes whose function is to catalyze the repair-replication of damaged DNA (1). This unscheduled DNA synthesis is crucial for the preservation of the structural integrity of DNA.

Hart and Setlow (2) used UV irradiation studies to show that those animals whose irradiated cells possess a greater capacity for DNA repair-replication have a longer life span. A different way of examining the validity of their observation is to determine both the levels of DNA repair enzyme activities and the genome integrity in the cells of aging animals. This problem can be more fruitfully investigated in post-mitotic cells such as neurons, muscle cells and heart cells (3). These nondividing cells need DNA repair enzymes to correct any damage that may occur in their DNA. The present communication reports the results which we obtained in our examination of the state of mouse cardiac cell DNA during senescence.

Materials and Methods

The mice used in this investigation were CBF males housed under conditions of three animals per cage. They were fed on antibiotic-free Agway diet specially formulated for Charles River Breeding Laboratories. Food and bedding were well sterilized. These mice have a mean life-span of 30±2 months.

Heart tissues were dissected out and collected in chilled 0.12 M sodium borate pH 8.5 and used for DNA preparation immediately or stored at -40°C. DNA was prepared from heart tissues of 6, 15, 20, 25, and 30 month old mice.
lysates were prepared by the method of Klotz and Zimm (4) which involved thoroughly suspending the pellet in 10 ml of boric acid buffer (pH 8.5) containing 0.18 M NaCl, 0.02 M disodium EDTA, 0.12 M boric acid, and 0.042 M NaOH. The suspension was mixed with 0.2 ml of 25% w/v sucrose, 0.2 ml of 25% w/v sarkosyl (K and K Fine Chemicals) and 5 mg of nuclease-free pronase (Calbiochem). The viscous DNA slurry was incubated at 68°C for 20 min, followed by several extractions with chloroform-phenol-isomyl alcohol (25:24:1) until the interphase was clear (5). DNA was precipitated with 95% ethanol, dissolved in 0.01 M NaCl, 0.01 M Tris-HCl (pH 7.5) and analyzed on CsCl gradients.

DNA was homogeneously mixed with CsCl to a final refractive index of 1.4001. The solution was centrifuged to equilibrium at 20°C for 40 hrs at 200,000 g (Spinco rotor SW 50.1). After collecting 0.3 ml fractions, and measuring the A_{260} and refractive index of each fraction, the fractions in the peak region were pooled, dialyzed against several changes of 100 volumes of 0.01 M Tris-HCl and stored at -20°C. The position of phage T₄ marker [³H]DNA was determined by measuring the amount of radioactivity in each fraction collected from the CsCl gradient.

The sedimentation of DNA was analyzed in 5-20% sucrose gradients made in 0.01 M EDTA, 0.4 M NaCl and 0.5 N NaOH (alkaline saline-EDTA) (6). About 15 μg of purified DNA were incubated in 0.5 ml of the above alkaline medium for 20 min at 22°C and then carefully loaded on top of the sucrose gradients. Centrifugation was at 10,500 rpm (rotor SW 27) for 18 hr at 4°C (7). The bottoms of the nitrocellulose tubes were punctured, 1.8 ml fractions collected and processed by the method of Siegel et al (8). [³H]Thymidine labelled mouse L1210 cell DNA (0.1-0.5 μg) was routinely sedimented in parallel gradients for comparison with the sedimentation of 15 μg heart DNA. Gradients were calibrated using T₄ phage DNA. The sedimentation coefficients based on the peaks of mouse DNA were determined relative to that of T₄ phage denatured DNA (57.5s) (9).

Sedimentation analysis and molecular weight calculations were carried out by the method of Peterson et al (10). The calibrated S_{20,w} was used to compute the molecular weight (M) by the equation of Studier (11); S_{20,w} = KM² where the constant K for denatured DNA in an alkaline medium was 0.0528 and the exponent α was 0.4. The method of Ehmann and Lett (12) was used to calculate the number average molecular weight (Mn) and the weight-average molecular weight (Mw) over the regions of the gradients encompassing the peaks. The fraction of the total A_{260} units of DNA on the gradient, that bands with a sedimentation coefficient corresponding to a molecular weight, M₁, was taken to be proportional to the mass of the DNA in that fraction by the relationship

\[ Mn = \Sigma \frac{A_{1}}{\Sigma (A_{1}/M_{1})} \]

and

\[ Mw = \Sigma \frac{M_{1}A_{1}}{\Sigma A_{1}} \]

where A₁ is the absorbance of a given fraction at 260 nm. The Mn of 6 month brain DNA and that of each peak-region of 30 month brain DNA was used to derive estimates of the number of single-strand breaks in the DNA of old mouse brain.

Nuclease S₁ was prepared by the method of Sutton (13) and used in determining the presence of single-stranded regions in brain DNA as described elsewhere (14). The nuclease S₁ preparations used in these studies did not digest double stranded DNA.

Results

The procedure used for cell lysis works best if cell suspensions devoid of connective tissue are used; this prevents the formation of lumps of tissue aggregates. Ravenoff and Zimm (15) used comparable lysis conditions in their
measurements of the relaxation times (and hence molecular weights) of cell lysate DNA, and found no evidence of the hydrolysis of the DNA by nucleases. We have found the combined use of brij 58 and sarcosyl to be essential for preventing a reduction in DNA viscosity during the 68°C incubation of the cell lysates.

The sedimentation of 6 month heart DNA is compared with that of phage T₄ DNA in Fig. 1A. Phage T₄ DNA (MW 1.2 x 10⁶ daltons) bands in the lighter region of the gradient (ρ = 1.7005) than mouse DNA which bands at a density of 1.701 g/cm³. These results corroborate those published by other investigators (16-18). A similar trend is also observed with 15 month heart DNA (Fig. 1B).

In both cases, it is quite clear that mouse DNA with 41% G+C (19,20) bands to the heavier side of T₄ DNA consisting of 34% G+C (16). The fact that this variance in buoyant density may be due in part to differences in G+C between phage T₄ and mouse DNA (21,22) invalidates the use of T₄ DNA as a molecular weight marker in these gradients. Furthermore it has been shown that obtaining meaningful molecular weights of DNA by sedimentation equilibrium in CsCl gradients requires running the gradients with several DNA concentrations which can be extrapolated to zero dilution (23). When 0.1 µg of labelled mouse L1210 cell DNA was centrifuged in parallel gradients, we found that it banded in the same region of the gradient as the 8 µg of the unlabelled heart DNA shown.
in Fig. 1, this observation indicates that the banding of heart DNA in the 1,701 g/cm³ region is not an artifact of overloading.

Fig. 2 shows the patterns of sedimentation of heart DNA isolated from 20, 25, and 30 month old mice. It can be observed that both the 20 and 25 month DNA samples band in broadened regions of the CsCl gradients (Fig. 2A). The density of a large component of each DNA sample is less than 1,701 g/cm³. The broadened bases of the peaks of both DNA samples now overlap the position of phage T₄ DNA. When the profile of the DNA from 20 month mice is compared with that of DNA from 25 month mice, the profile of the latter appears to be distributed over a broader region of the gradient.

![Fig. 2: Sedimentation profiles of heart DNA from 20, 25 and 30 month old mice. Sedimentation is from right to left. Gradients are calibrated using T₄ phage DNA as marker shown by lower arrow. Density, ρ; 20 month (O), and 25 month (●) (A); 30 month (B).](image)

The DNA from the hearts of 30 month old mice (Fig. 2B) sediments in an even broader region of the gradient than the DNA isolated from 25 month old mice. The broadening of the sedimentation profile of 30 month heart DNA is such that a larger component of the DNA now bands in the position occupied by phage T₄ DNA. The significance of this skewed mode of sedimentation remains unclear. These results show that the DNA from the hearts of aging mice becomes smaller in molecular weight than the DNA isolated from young mice. It seems that heart DNA develops breaks as the mouse ages.
TABLE 1
Endonuclease Digestion of Mouse Heart DNA

<table>
<thead>
<tr>
<th>Ages of mice (months)</th>
<th>% DNA digested*</th>
<th>native</th>
<th>denatured</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>2.6±0.6</td>
<td>96±2</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>12.8±0.4</td>
<td>94±2</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>15.1±0.4</td>
<td>95±2</td>
<td></td>
</tr>
</tbody>
</table>

*Each value is based on 3 experiments

We further analyzed heart DNA to determine if it also develops single-stranded gaps with advancement in age. Table 1 summarizes the results obtained when DNA from 6, 20, and 30 month old mice was treated with single-strand specific S<sub>1</sub> endonuclease. The results show that the enzyme digests only 2.6% of 6 month heart native DNA as compared to its digestion of 15.1% of the native DNA from 30 month mouse hearts. In both cases it can be seen that denatured DNA from both 6 and 30 month old mice is almost completely digested by S<sub>1</sub> endonuclease.

To determine the molecular weights and the nature of breakdown occurring in the DNA, we analyzed 6 and 30 month heart DNA on alkaline sucrose gradients. The sedimentation profiles of these two DNA samples and that of [<sup>3</sup>H]DNA from mouse L1210 cells are shown in Fig. 3. It can be observed that the 30 month heart DNA bands in three regions of the gradient. Peak 1 fraction (2 x 10<sup>6</sup> daltons) bands in the heavier region of the gradient, followed by peak 2 (6.5 x 10<sup>6</sup> daltons), and finally peak 3 (3 x 10<sup>6</sup> daltons). These peaks are highly reproducible. We used the Mn of the pieces of DNA contained in each peak to calculate the number of single strand breaks that must occur in peak 1 DNA in order for it to give rise to the fractions in peaks 2 and 3. The calculations show that peak 2 fraction results from the equivalent of two evenly spaced breaks and peak 3 from six breaks in peak 1 DNA. These estimates of the number of single strand breaks probably represent an underestimate of the actual degree of breakage in the DNA of mammalian chromosomes whose molecular weight is estimated to be in excess of 10<sup>9</sup> - 10<sup>10</sup> daltons (24, 25).

It will also be noted that the DNA of L1210 cells bands in the peak 1 region. The possible role of putative old age specific nucleases in degrading old mouse DNA during isolation has been investigated by mixing L1210 cell [<sup>3</sup>H]DNA with heart cell lysates during the 20 min incubation at 68°C preceding DNA purification and alkaline sucrose gradient sedimentation. L1210 cell DNA continued to band in the peak 1 region as in Fig. 3.

Discussion

One reason for our interest in examining the state of the structure of old mouse DNA is because senescence has been shown to be causally associated with the loss of certain genes (26). Our results provide evidence that shows an age-associated fragmentation of mouse heart DNA. It is conceivable that the light sedimenting DNA could represent those portions of the genome that are eventually lost in old age (26). The progressive degradation of the DNA suggested by both sedimentation profiles and S<sub>1</sub> nuclease assays suggests either a greater accumulation of DNA-digesting enzymes or a decline in the repair-replication capabilities of old heart cells, or both (2, 27).

The broadening of the sedimentation profile of old heart DNA in CsCl density gradients is known to be a function of molecular weight heterogeneity (21,22), while the shift in banding position is determined by its base composition (22).
or molecular configuration (28). The reason why the sedimentation profiles of old heart DNA are skewed toward the light side of the gradient (Fig. 2) is unclear, unless there is an age-associated preferential loss of G + C rich regions in old heart DNA. Such a loss of G + C rich portions of the genome, if it could be substantiated, is not without precedence. Johnson and Stehler (26) have reported a loss of the G + C rich ribosomal DNA sequences in the brain cells of old beagles.

Fig. 3
Alkaline sucrose gradient profiles of old heart DNA suggest that it contains single-stranded gaps (Fig. 3). Part of the evidence for this notion comes from the fact that these pieces of DNA are substantially digested by single strand specific S1 endonuclease as well as the sedimenting of the DNA in three discrete peaks under the denaturing conditions in alkaline sucrose gradients. Fig. 4 presents a postulated model of the state of old heart DNA molecules which when sedimented under denaturing conditions, would give the sedimentation profiles shown in Fig. 3. The material sedimenting in peaks 2 and 3 would come from molecules of the type depicted in Fig. 4A, while the material in peak 1 would come from the type of molecule in Fig. 4B. There is a possibility that part of the fragmentation of senescent mouse DNA is generated by mechanical shear in the single-stranded gaps during DNA purification. This notion derives support from the results of Abelson (29).
who showed that DNA with more single-stranded discontinuities is more sensitive to mechanical shear than molecules of equivalent length containing fewer discontinuities.

![Diagram](image.png)

Fig. 4

Postulated structures of DNA in old mouse neurons. (A) DNA molecule with 4 regions with single strand breaks subject to nuclease attack, and 4 duplex regions; 5 single strands would sediment in alkaline sucrose gradients before nuclease attack and probably 8 strands after nuclease digestion of single-stranded regions. (B) DNA with one single-stranded stretch at right terminus (region b).

Recently Lampidis and Schaiberger (30) have demonstrated an age-related loss of DNA repair-replication in myocardial cells isolated from adult rats; even after UV irradiation, they could not detect the expected repair of DNA (31). They interpreted this as indicating that repair enzyme activities decline as the heart cells of growing mice enter the non-proliferative phase. If this interpretation is correct, one can extrapolate this reduction in repair enzyme activity in post-mitotic heart cells to the senescent stage of the organisms, and attribute the age-associated breakdown of myocardial cell DNA to deficiencies in repair enzyme activities.

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References