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<td>Author(s)</td>
<td>Kiyoto, Shinkai</td>
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Radiation and Ageing
An Electron Microscopic Observation and
Morphometrical Study on Lipofuscin in the
Cerebellar Purkinje Cells, Myocardial Cells and Hepatic Cells

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SUMMARY

Four-week old and twelve-week old female Wistar rats were used in a long-term survival experiment undertaken as an investigation of ageing in the living body. Lipofuscin in the Purkinje cells, myocardial cells and hepatic cells of the rats was observed electron microscopically, and ageing in the living body was investigated by morphometrical analysis of lipofuscin. At the same time, the acceleration of ageing due to irradiation was investigated. As a result, the accumulation of lipofuscin with ageing in Purkinje cell and myocardial cell corresponding to a post-mitotic cell was shown in both irradiated and control groups. For that reason, it was conjectured that lipofuscin can be regarded as a morphological marker, although the accumulation of lipofuscin in hepatic cells having high regeneration potential was not shown. Therefore, it is difficult to regard lipofuscin in hepatic cells as a morphological marker. Comparing the 300R irradiated group with control group, a significant difference ($p<0.05$, $p<0.01$) concerning accumulation of lipofuscin was
shown in only Purkinje cells. The correlation between accumulation of lipofuscin and ageing in 300R irradiated groups showed a more significant difference (p<0.05) than that in control groups, on the basis of regression lines calculated in the three kinds of cell.

INTRODUCTION

The mechanism of ageing in the living body is still not fully elucidated, but many investigations and theories on ageing have been presented. Ageing is likely caused by interaction among numerous complex mechanisms. Many investigations concerning the shortening of life span and acceleration of ageing in atomic bomb survivors have been performed over the years in both Hiroshima and Nagasaki. Nevertheless, no definite conclusion has been reached because the influence of exposure to radiation is probably masked by various environmental, that is, chemical, physical and psychological, factors. The author paid much attention to the theory of free--radical due to irradiation, and observed lipofuscin (Lf), which is one of the morphological markers, electron microscopically. The author then performed a morphometrical analysis, and investigated ageing and its acceleration in the living body.

MATERIALS AND METHODS

Forty-four 4-week old and forty-four 12-week old female Wistar rats were prepared as experimental animals. Twenty four rats were selected from each group at random and irradiated with doses of 150R for four-week old rats and 300R for twelve-week old rats. The remaining rats were used as controls (Table 1). A TOSHIBA KXC-19-7 was used as an X-ray irradiation apparatus. The conditions of irradiation were 200kvp in voltage, 20mA in current, Filter 0.5Cu + 0.5Al, and distance from focus was 107.5cm and 105.5cm for 150R and 300R, respectively. A VICTREEN-555 was used as a dosimeter. After irradiation, the general condition of the rats was observed every day and body weight was measured every month. The rats were sacrificed by decapitation two, four, twelve, and eighteen months post--irradiation. A small portion of the cerebellum, heart and liver was removed, immediately dripped with 1.5% glutaraldehyde, cut in slender pieces and then fixed in 1.5% glutaraldehyde. After fixation in osmium tetroxide the specimens were dehydrated in alcohol series and embedded in Luveak-812. The semithin sections were
stained with toluidine blue. The ultrathin sections were double stained with uranyl acetate and lead citrate, and then observed using a HITACHI-600 electron microscope. Cerebellar Purkinje cells (P-cell), myocardial cells (M-cell) and hepatic cells (H-cell) were observed, and then photographs of 30 to 36 cells with identified nuclei from each group were taken. The areas of both cytoplasm and Lf, and the number of Lf were measured. The percent area of Lf with respect to cytoplasm was also calculated using the following equation: percentage of area occupied by Lf (%) = \[ \frac{\text{Area of Lf} (\mu m^2)}{\text{Area of cytoplasm} (\mu m^2)} \] \times 100. The final magnifications on electron micrographs were x 3,000 and x 10,000, respectively. For measurement the modular system for semiautomatic quantitative evaluation images of A. S. M. LEITZ, West Germany, and statistical procedure IBM-4361 programed BMDP (made at UCLA) were used and produced the display of regression lines and the paired t-test.

RESULTS

General and Macroscopic Findings

Some of the irradiated rats suffered from mild to moderate diarrhea for one or two days post-irradiation, but recovered within one week. None of the rats had a bloody stool. Epilation was observed in 150R and 300R irradiated groups from two months post-irradiation, 0.09% and 0.17%, respectively (Table 2). Post-irradiation body weight in the 150R irradiated groups showed a significant decrease (p > 0.05) in comparison with that in the control groups until four months post-irradiation (Fig. 1). No significant difference between the 300R irradiated groups and the control groups was acknowledged in post-irradiation body weight (Fig. 2). Similarly, the weight of the brain, heart and liver

<table>
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<th>Initiated Age</th>
<th>Number</th>
<th>Dose</th>
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<tr>
<td>4W</td>
<td>24</td>
<td>150R</td>
</tr>
<tr>
<td>4W</td>
<td>20</td>
<td>Control #-1</td>
</tr>
<tr>
<td>12W</td>
<td>24</td>
<td>300R</td>
</tr>
<tr>
<td>12W</td>
<td>20</td>
<td>Control #-2</td>
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Table 2. General Condition

<table>
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<tr>
<th>Treatment</th>
<th>Diarrhea</th>
<th>Epilation</th>
</tr>
</thead>
<tbody>
<tr>
<td>150R</td>
<td>15/24 (62.5%)</td>
<td>2/24 (0.83%)</td>
</tr>
<tr>
<td>Control #1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>300R</td>
<td>20/24 (83.3%)</td>
<td>4/24 (0.17%)</td>
</tr>
<tr>
<td>Control #2</td>
<td>-</td>
<td>-</td>
</tr>
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</table>

showed no significant difference between irradiated and control groups (Table 3).

*Light Microscopic Findings*
Light microscopically it was difficult to observe Lf in P-cell, M-cell and H-cell eighteen months post-irradiation, using Hematoxilin-Eosin (H. E.) staining. A few Lfs were shown as P. A. S. -positive granules in P-cell and M-cell twelve and eighteen months post-irradiation, using Periodic Acid Schiff (P. A. S.) staining. Lf in H-cell, however, was not displayed by P. A. S. staining due to rich glycogen particles.

*Electron Microscopic Findings*
Lfs were observed in the perikaryocyte of P-cells (Fig. 3), and in the cytoplasm near the main dendrite in some P-cells. Each Lf of the old rats increased in size and had a more irregular configuration in comparison with young rats (Fig. 4). Sometimes Lfs with small lipid droplets were also observed. No morphological differences of Lf were found between irradiated and control groups.
Fig. 3b (right). Higher magnification of Fig. 3a. Lipofuscin's have a irregular configuration and a small vacuolation.

original magnification X 5,000

Fig. 3a (left). Purkinje cell of four months post-irradiation. A few small lipofuscin's are seen in the cytoplasm near the main dendrite.

original magnification X 1,500

(M-cell)

Numerous mitochondria were densely observed on both sides of the elongated nucleus. Lfs were observed among them (Fig. 5a). Lfs were observed not only on both sides of the nucleus, but also on the unilateral side of the nucleus. Lfs of old rats increased in size and had a more irregular configuration in comparison with young rats (Fig. 5b). Intracytoplasmic organellas such as endoplasmic reticulum and Golgi apparatus etc. were observed scarcely. No morphological differences of Lf were found between irradiated and control groups.

(H-cell)

Rich glycogen particles, numerous mitochondria, endoplasmic reticulae and Golgi apparatus etc. were observed in the cytoplasm. Lfs were scattered among them. Lfs in the H-cells were relatively small and round, and Lfs with electron low density were frequently observed in both young and old rats (Fig. 6). No morphological differences of Lf were found between irradiated and control groups.

Morphometrical Analysis
Fig. 4a (left). Purkinje cell of eighteen months post-irradiation. Several large lipofuscins are shown in the cytoplasm.

original magnification X 1,500

Fig. 4b (right). Higher magnification of Fig. 4a. Lipofuscin with vacuolation, low and high density increase in size and number, and have more irregular configuration than that of young rats.

original magnification X 5,000

Fig. 5a. Myocardial cell of two months post-irradiation. Lipofuscin is displayed in numerous mitochondria adjacent to the pole of the elongated nucleus.

original magnification X 5,000
Fig. 5b. Myocardial cell of eighteen months post-irradiation. Lipofuscin increase in size and number, and have more irregular configuration than that of young rats.

original magnification X 5,000

(Relationship between ageing and Lf)
A) Statistical analysis

The area, number and percent area of Lf increased in both irradiated and control groups of P-cells and M-cells (Table 4-7), but there was no correlation between accumulation of Lf and ageing in H-cell (Table 8,9). The area of the three kinds of cell showed no change with ageing.

B) Regression line

The area, number and percent area of Lf showed a strongly positive correlation (p<0.05) with respect to ageing in both irradiated and control groups of P-cells and M-cells (Fig. 7,8). However, the above correlation was not acknowledged in either group of H-cell (Fig. 9).

(Relationship between irradiation and Lf)
A) Statistical analysis

The area, number and percent area of Lf in 300R irradiated groups showed a statistically significant difference (p<0.05) when compared with control groups two, four,
Fig. 6. Hepatic cell of twelve months post-irradiation. A few small and round lipofuscins (arrowheads) are scattered among the rich glycogen particles and the numerous rough E. R. etc.
original magnification X 5,000

Table 4. P-cell in 150R.

<table>
<thead>
<tr>
<th>Post-Irradiation</th>
<th>2M</th>
<th>4M</th>
<th>12M</th>
<th>18M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area of cytoplasm</td>
<td>315.8±11.58</td>
<td>357.0±13.71</td>
<td>368.5±14.47</td>
<td>284.1±12.71</td>
</tr>
<tr>
<td>(um²)</td>
<td>319.7±13.53</td>
<td>329.1±11.72</td>
<td>352.6±13.58</td>
<td>254.3±8.78</td>
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<tr>
<td>Area of Lf</td>
<td>2.27±0.26</td>
<td>2.72±0.28</td>
<td>4.29±0.48</td>
<td>4.59±0.54</td>
</tr>
<tr>
<td>(um²)</td>
<td>2.29±0.23</td>
<td>2.41±0.26</td>
<td>4.00±0.52</td>
<td>5.39±0.79</td>
</tr>
<tr>
<td>Number of Lf</td>
<td>3.78±0.36</td>
<td>5.89±0.48</td>
<td>6.50±0.49</td>
<td>8.78±0.85</td>
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<tr>
<td>150R.</td>
<td>Control-1</td>
<td>3.36±0.29</td>
<td>4.33±0.35</td>
<td>5.46±0.05</td>
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<tr>
<td>Control-1</td>
<td>10.07±1.27</td>
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<tr>
<td>Percent area of Lf</td>
<td>0.73±0.08</td>
<td>0.77±0.08</td>
<td>1.15±0.13</td>
<td>1.65±0.17</td>
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<tr>
<td>150R.</td>
<td>Control-1</td>
<td>0.71±0.06</td>
<td>0.74±0.08</td>
<td>1.15±0.16</td>
</tr>
<tr>
<td>Control-1</td>
<td>2.13±0.31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean±S.E.</td>
<td><strong>p&lt;0.05</strong></td>
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</table>

Table 5. P-cell in 300R.

<table>
<thead>
<tr>
<th>Post-Irradiation</th>
<th>2M</th>
<th>4M</th>
<th>12M</th>
<th>18M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area of cytoplasm</td>
<td>260.96±8.74</td>
<td>325.76±14.77</td>
<td>331.62±11.06</td>
<td>300.99±11.56</td>
</tr>
<tr>
<td>(um²)</td>
<td>257.86±12.53</td>
<td>314.20±13.91</td>
<td>336.11±14.38</td>
<td>310.26±13.61</td>
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<tr>
<td>Area of Lf</td>
<td>3.38±0.29</td>
<td>3.88±0.43</td>
<td>7.07±0.86</td>
<td>6.03±0.93</td>
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<tr>
<td>(um²)</td>
<td>2.14±0.28</td>
<td>2.84±0.30</td>
<td>4.22±0.49</td>
<td>4.71±0.68</td>
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<tr>
<td>Number of Lf</td>
<td>4.57±0.35</td>
<td>6.44±0.58</td>
<td>9.61±1.22</td>
<td>8.67±1.01</td>
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<tr>
<td>300R.</td>
<td>Control-2</td>
<td>3.07±0.31</td>
<td>4.77±0.52</td>
<td>6.70±0.93</td>
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<tr>
<td>Control-2</td>
<td>Mean±S.E.</td>
<td>*p&lt;0.05</td>
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<tr>
<td>Percent area of Lf</td>
<td>1.32±0.11</td>
<td>1.20±0.13</td>
<td>2.15±0.25</td>
<td>1.91±0.28</td>
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<tr>
<td>(um²)</td>
<td>0.84±0.10</td>
<td>0.87±0.08</td>
<td>1.32±0.19</td>
<td>1.51±0.20</td>
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<tr>
<td>300R.</td>
<td>Control-2</td>
<td>Mean±S.E.</td>
<td><strong>p&lt;0.01</strong></td>
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and twelve months post-irradiation in P-cells. However, the above difference was not observed in any cells of the 150R irradiated groups (Table 4,5). No difference between irradiated and control groups of 150R and 300R experimental groups was observed in M-cells and H-cells (Table 6-9).
B) Test of equality of lines between irradiated and control groups

With regard to each regression line of the area, number and percent area of Lf with respect to ageing, the correlation of 300R irradiated groups showed a highly significant difference ($p < 0.05$) compared with that of control groups (Fig. 7–9). However, the above difference was not observed between 150R irradiated and control groups.
DISCUSSION

There have been many investigations, both in vivo and in vitro, on disease due to irradiation. Irradiation causes the occurrence of malignancies and cataract, detrimental hereditary influences and shortening of life span. Especially with respect to the latter, it is a well-known fact that irradiation causes the shortening of life span in animal experiments\textsuperscript{2,5,6,16}. However, the shortening of life span in humans has not been proven because it is difficult to keep various conditions constant\textsuperscript{11,21}. The LD\textsubscript{50} (X-ray) of the Wistar rat is approximately 600R\textsuperscript{4}, but due to the long period of observation in the present study doses of 150R and 300R were established.

Hursh \textit{et al.}\textsuperscript{13} irradiated Wistar rats with 150R-600R doses, and calculated the ratio of shortening of life span. According to them, 150R and 300R doses shortens the life span by 4.2\% and 23.8\%, respectively. The same ratio of shortening of life span was expected in this series of experiments, and the experiments performed in order to observe the changes with ageing in surviving rats.

Since Hannover\textsuperscript{8} found Lf in the nerve cells of the senile brain, Lf has received much attention as one of the morphological markers of ageing, and many related investigations have been carried out\textsuperscript{3,7,10,11,25}. Light microscopically, Lf is observed widely in the muscles, liver, kidney, adrenal gland and nerve cells etc. as yellow or yellow-brown pigments (H. E. staining). Electron microscopically, Lf has an irregular configuration and three components: 1) electron-lucent homogenous droplets; 2) granular matrix; 3) osmiophilic...
patches\(^{(10)}\). However, it is a fact that some Lfs lack the first component which is expected to contain a lipid. It is also a well-known fact that post-mitotic cells such as, muscle and nerve cells etc. accumulate Lf with ageing\(^{(7,10,11)}\). Light microscopically, a small number of Lfs can be observed clearly in the nerve cells of humans in the second decade\(^{(14)}\). KISHIKAWA\(^{(11)}\) stated that Lfs were already present in the fetal dorsal root ganglia, electron microscopically.

Originally, P-cell was considered to be a nerve cell with scarce Lf\(^{(6,19)}\), but electron microscopically it has a few Lfs. As shown in the experimental data (Table 4), Lf increased with ageing in P-cells, just as in other nerve cells. A markedly positive correlation was observed between the increase of Lf and ageing, according to regression lines (Fig. 7). This means that, in P-cell as well, Lf can be regarded as a morphological marker of ageing. OGATA et al.\(^{(20)}\) also performed electron microscopic and morphometrical analysis of Lf in the P-cell of rats, and investigations concerning ageing. Their statement that Lf is useful as a morphological marker of ageing corresponds with the present experimental data (Table 4).

M-cell is also one of the post-mitotic cells, and accumulation of Lf with ageing was observed, just as in P-cell (Table 6,7). A positive correlation was observed between accumulation of Lf and ageing (Fig. 8), that is, it was acknowledged that Lf can be regarded as a morphological marker, just as in P-cells.

In the case of H-cells, no correlation whatsoever was observed between Lf and ageing (Fig. 9). SATO et al.\(^{(23)}\) observed Lf in human H-cells with ageing but doubted that Lf in H-cell could be regarded as a morphological marker of ageing. H-cell has high regenerative potential because it is not a post-mitotic cell like P-cell and M-cell. For that reason, it is supposed that although Lf is produced in H-cell it is does not accumulate there, but is disposed of by excretion or by active metabolism.

Ageing is one of the important factors in the accumulation of Lf. SULKIN et al.\(^{(24)}\) experimentally proved that hypoxic state, vitamine-E deficiency, acetooanilide administration, and long-term ACTH administration after unilateral nephrectomy also caused the accumulation of Lf.

At present, it is the most popular theory that, ultrastructurally and histochemically, Lf originates from primary lysosome\(^{(10,12,22)}\). Some investigators, however, insist on the mitochondrial origin of Lf\(^{(25)}\). The author frequently observed primary lysosome adjacent to Lf in each kind of cell, and some Lfs could not be clearly distinguished from primary lysosomes. This fact suggests that primary lysosome may gradually alter to Lf, and that Lf has an intimate relationship with primary lysosome.
Some current investigations present the relationship between Lf and lipid peroxide (LPX) in the living body\textsuperscript{2,26).} YOSHIKAWA et al\textsuperscript{26) proved that LPX in the living body increased with ageing. LPX is considered to be a precursor in the productive process of Lf. LPX is formed from unsaturated lipid acid, which comprises the membrane of intracytoplasmic mitochondria, microsome and other organelles, and in process LPX causes protein degeneration. Consequently, it seems that LPX combines with degenerative protein and this polymer is taken into primary lysosome, thereby producing Lf\textsuperscript{12).}

Irradiation is one of the factors that causes the alteration of LPX from unsaturated lipid. In fact, it has already been proved that LPX increases due to irradiation of the living body\textsuperscript{2,9).} In the present series of experiments, a statistically significant difference (p<0.05, p<0.01) was shown in the P-cells of the 300R irradiated groups. According to the test of equality of regression lines, the correlations among 300R irradiated groups were statistically significant (p<0.05) compared with the control groups in the three kinds of cell (Fig. 7–9). This analysis suggests that accumulation of Lf in the three kinds of cell shows a marked correlation with ageing due to 300R irradiation. In other words, the present study suggests that ageing is accelerated by irradiation from the point of view of the morphological analysis of Lf.

**CONCLUSION**

Electron microscopically, the author observed Lf, which is one of the morphological markers of ageing, in P-cells, M-cells and H-cells, in order to investigate ageing in the living body and acceleration of ageing due to irradiation. Furthermore, the following conclusions were arrived at on the basis of morphometrical analysis of Lf.

1) In investigating the acceleration of ageing due to irradiation, a statistically significant difference (p<0.05, p<0.01) was observed between irradiated and control groups only in the P-cells in the 300R experimental group.

2) Regression lines revealed that the correlation of irradiated groups between accumulation of Lf and ageing was more significant (p<0.05) than that of controls in the three kinds of cell. This fact shows that there is a marked correlation between accumulation of Lf and ageing due to 300R irradiation. There was no difference between the two groups in the 150R experimental group.
3) It is interesting that the present investigation uses the morphological marker Lf to show that irradiation causes the acceleration of ageing, as discussed in 1) and 2).

4) Lf can be regarded as a morphological marker of ageing in P-cells and M-cells. However, it is difficult for Lf in H-cell with high regeneration potential to be regarded as a morphological marker of ageing.

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