D-Aspartic Acid in Aged Mouse Skin and Lens

NORIKO FUJII¹, ITSURO TAMANOI², SHIRO MURAOKA⁴, HISAMASA JOSHIMA³, MASATOSHI KASHIMA⁴ and KAORU HARADA¹

¹Department of Chemistry, University of Tsukuba, Niihari, Ibaraki 305, Japan
²Biological Department, College of Arts and Sciences, Chiba University, Yayoi-cho, Chiba 260, Japan
³Training School, National Institute of Radiological Sciences, Anagawa 4, Chiba 260, Japan
⁴Division of Radiation Hazards, National Institute of Radiological Sciences, Anagawa 4, Chiba 260, Japan

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D-aspartic acid (D-Asp) was detected in the skin and lens from naturally aged mice. An analysis of the amino acid composition indicated that D-Asp did not derive from collagen. An immunological analysis using Ouchterlony’s agar diffusion method also confirmed that the protein containing D-Asp was not a serum protein. The process producing D-Asp is regarded as one other than racemization because the life span of mice is not long enough to permit D-Asp by racemization. Continuous low-dose-rate gamma-irradiation (37R per day) for 102 to 112 days did not increase significantly the amount of D-Asp in skin and lens of mice.

INTRODUCTION

Proteins have been considered to consist exclusively of L-amino acids. Recently, however, D-aspartic acid (D-Asp) has been detected in various human tissues such as tooth¹,², eye lenses³-⁵ and white matter of brain⁶, in samples from elderly individuals. It has been explained that the presence of D-Asp is the result of racemization of aspartyl residues in the polypeptide chain during the life span, inasmuch as the proteins in these tissues are metabolically inert. However, the white matter of brain is different from tooth or lens and is metabolized rather rapidly. D-Asp has also been found in erythrocyte membrane protein⁷ which is a metabolically active protein.

Our previous study reported the presence of D-Asp in the cataract lenses of aged mice⁸. Three months old mice were totally irradiated with a sub-lethal dose by ¹³⁷Cs and maintained for 18 months. The amount of D-Asp was not affected directly by radiation, but in the i-
radiated mice increased with aging. If the formation of D-Asp was caused by racemization only, the life span of mice would be too short to allow D-Asp to accumulate by racemization. Thus, it is not clear at this time how D-Asp in protein is formed in the short life span. In this report the presence of D-Asp in the skin and lens from naturally aged mice and also from mice that had been raised under continuous exposure to low dose-rate gamma-rays, was investigated.

MATERIALS AND METHODS

Animals and Irradiation

Male and female mice of C57/HHe, (ICR/JCL x C57BL/6J) F1 and BALB/cA strains were used. The former strain and hybrid mice served both as the naturally aged (20–30 months old) and the younger control (1–3 months old). BALB/cA mice, 3 months old, were subjected continuously to whole body irradiation with 37R/day (22 h) of gamma-rays from $^{137}$Cs for 102–112 days. The irradiated mice were sacrificed immediately after termination of the irradiation.

Preparation of protein from skin and lenses

The proteins from skin or lenses were obtained in the same way as previously described. Fresh mouse skin was freed from adherent tissues such as fatty materials and hair, and cut into small pieces with scissors and homogenized with a homoblender and a glass homogenizer in an ice bath. The resulting material was washed with chilled water and then extracted 2 times with 5 volumes of 0.15 M NaCl solution. The residue was resuspended and reextracted twice with 5 volumes of 0.45 M NaCl. The extracts of these fractions were dialyzed against distilled water and lyophilized.

A lens was homogenized with a glass homogenizer in 1 ml of cold distilled water and fractionated into water soluble (WS) and water insoluble (WI) fractions by centrifugation.

An aliquot of the extract with 0.45 M NaCl from skin or WI fraction of lens was dissolved in water and fractionated by adding gradually cold EtOH while kept on an ice bath. The final concentration of EtOH was adjusted to 60%. After stirring, the sample solution was allowed to stand for 30 min at 4°C, and then centrifuged at 5000 g for 15 min. The supernatant (60% EtOH sup fraction) and precipitate (60% EtOH ppt fraction) were dried.

Determination of the D/L ratio of the amino acid

All glassware was baked at 500°C for 4 h in a furnace before use. The sample was hydrolyzed with 6 N HCl at 110°C for 22 h in an evacuated sealed tube. The hydrolyzate was dried under reduced pressure and the residual amino acids were converted to N-trifluoroacetyl amino acid isopropyl esters in the usual manner. The ratio of D-to L-amino acid was determined by a gas-liquid chromatography (Hitachi 163 Gas Chromatograph) using a chiral capillary column (Chirasil-Val 25 m x 0.25 mm, Applied Science, USA).

The determination of amino acid composition

After hydrolysis and drying of the samples, the composition of residual amino acids was determined with an amino acid auto analyzer, Dionex Model D-502.
**Immunological analysis of serum components by agar diffusion**

Proteins extracted from the skin were analyzed by Ouchterlony's agar diffusion method\(^1\). Antisera used were rabbit antisera against mouse serum and serum albumin. Rabbit anti-mouse serum antiserum was purchased from Miles-Yeda Ltd. (Lot No. R 484). Antiserum against mouse serum albumin was produced by intramuscular injection of serum albumin with Freund's complete adjuvant (Difco) into rabbits. Serum albumin was collected from pooled mouse sera by salting out with ammonium sulfate and then fractionated by column chromatography using DEAE-Sephadex A-50 (Pharmacia).

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**Fig. 1. Gas Chromatograms of N-trifluoroacetyl isopropyl esters of amino acids from hydrolysate of the protein which were obtained by the treatment with 60% EtOH of the 0.45 M NaCl fraction from naturally aged mouse skin. ((ICR/JCL x C57BL/6J) F1; 28 months old)**

(A) 60% EtOH sup fraction
(B) 60% EtOH ppt fraction
(a part of gas chromatogram)
RESULTS

Determination of the D/L ratio of aspartic acid in the protein extracted from skin and lenses

The D/L ratio of aspartic acid in the isolated proteins was determined by a gas liquid chromatography. An example of gas chromatogram for the 60% EtOH sup and ppt fractions of skin from aged mouse is shown in Fig. 1. The higher concentration of D-Asp was obtained from the 60% EtOH sup fraction, but not from the ppt fraction of the aged mouse skin. Neither the 60% EtOH sup nor ppt fraction from younger mouse skin showed such a high value. The D/L ratio of aspartic acid in these skin fractions from the other aged and younger mice are summarized in Table 1. A high D/L ratio of aspartic acid was detected in EtOH sup fractions of 4 among 9 aged skin samples irrespective of the strain and sex of the mouse. The D/L ratio of aspartic acid of all the younger mouse samples (control) was the average value of 20 younger mice and the deviation was within ±0.003.

The WI fraction of lenses from aged mice contained a slightly higher amount of D-Asp. Thus, WI fractions of lens were treated with 60% EtOH and the D/L ratio of aspartic acid ana-

| Mouse | Sex a) | Age (Months) | 60% EtOH sup Fr. | 60% EtOH ppt Fr. |
|-------|--------|--------------|-----------------|-----------------|
| (ICR/JCL × C57BL/6J) F1 | F | 20 | 0.099 | 0.056 |
| (ICR/JCL × C57BL/6J) F1 | F | 27 | 0.117 | 0.060 |
| (ICR/JCL × C57BL/6J) F1 | F | 28 | 0.093 | 0.030 |
| C3H/He | M | 30 | 0.090 | 0.056 |
| (ICR/JCL × C57BL/6J) F1 | F | 28 | 0.041 | 0.039 |
| (ICR/JCL × C57BL/6J) F1 | M | 28 | 0.034 | 0.043 |
| (ICR/JCL × C57BL/6J) F1 | M | 28 | 0.057 | 0.044 |
| (ICR/JCL × C57BL/6J) F1 | F | 29 | 0.038 | 0.044 |
| C3H/He | M | 32 | 0.057 | 0.036 |
| C3H/He (control) b) | M | 1 | 0.033 | 0.039 |

a) F: female, M: male
b) average value

| Mouse | Sex a) | Age (Months) | 60% EtOH sup Fr. | 60% EtOH ppt Fr. |
|-------|--------|--------------|-----------------|-----------------|
| (ICR/JCL × C57BL/6J) F1 | F | 20 | 0.069 | 0.055 |
| (ICR/JCL × C57BL/6J) F1 | F | 26 | 0.066 | 0.054 |
| (ICR/JCL × C57BL/6J) F1 | M | 27.5 | 0.071 | 0.053 |
| C3H/He | M | 30 | 0.070 | 0.055 |
| C3H/He | M | 30 | 0.080 | 0.062 |
| C3H/He | M | 30 | 0.080 | 0.056 |
| C3H/He | M | 30 | 0.104 | 0.047 |
| C3H/He (control) b) | M | 1 | 0.044 | 0.046 |

a) F: female, M: male
b) average value
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lyzed in the same manner as for skin. The results are shown in Table 2. The 60% EtOH sup fraction of lens from the aged mice always showed a higher D/L ratio of aspartic acid than that from younger mice.

Effect of low dose continuous irradiation on the D/L ratio of proteins extracted from skin and WI fraction of lenses

The effect of continuous gamma-irradiation on the presence of D-Asp in skin and lenses was investigated. The D/L ratios of aspartic acid in skin or lenses from the irradiated and the control mice are shown in Table 3. No significant difference in the D/L ratio of aspartic acid in either skin and lens was observed even after a long period of continuous irradiation (for 112

| Table 3. | D/L ratio of aspartic acid of skin (0.45 M NaCl extracts) and lens (WI fraction) after continuously irradiation (37R/day). |
|--------------------------------------------------|
| Irrad. (days) | Sex | Skin (0.45 M NaCl Fr.) | Lens (WI Fr.) |
|----------------|-----|-----------------------|----------------|
| 102            | M   | 0.045                 | 0.051          |
| 105            | M   | 0.051                 | 0.054          |
| 109            | M   | 0.049                 | 0.051          |
| 112 control(1) | M   | 0.050                 | 0.053          |

a) M: male
b) average value

| Table 4. | Amino acid composition of protein of aged mouse skin (mouse: (ICR/JCL x C57BL/6J) F1, 27 months old, D/L ratio of Asp of 60% EtOH sup fraction is 0.117) |
|----------------|---------------------------------|
| Amino acid | 60% EtOH sup | 60% EtOH ppt |
| Asp        | 69               | 41           |
| Hyp        | 0                | 55           |
| Thr        | 56               | 40           |
| Ser        | 74               | 51           |
| Glu        | 165              | 115          |
| Pro        | 36               | 78           |
| Gly        | 172              | 200          |
| Ala        | 111              | 100          |
| Val        | 54               | 45           |
| Cys        | 13               | 5            |
| Met        | 9                | 9            |
| Ile        | 34               | 33           |
| Leu        | 63               | 62           |
| Tyr        | 21               | 17           |
| Phe        | 25               | 25           |
| Hyl        | 0                | 7            |
| Lys        | 64               | 52           |
| His        | 11               | 13           |
| Arg        | 23               | 52           |

Res/1000 Res
day with 37R/day, totaling over 4000R). The 0.45 M NaCl fraction of the skin and WI fraction of lenses after continuous irradiation were treated with 60% EtOH solution. However, a higher D/L ratio of aspartic acid was detected neither in the sup nor in the ppt fraction of 60% EtOH solution.

Analysis of amino acid composition of protein extracted from aged mouse skin

The amino acid composition of the EtOH sup and ppt fractions from aged mouse skin is shown in Table 4. The D/L ratio of aspartic acid in the EtOH sup fraction from aged mouse skin was 0.117. Hydroxyproline and hydroxylysine were not detected in this 60% EtOH sup fraction, but were detected in the 60% EtOH ppt fraction. The protein content (total amino acid) in the EtOH sup fraction was about 1/5 of its corresponding ppt fraction.

Immunological analysis of serum components in extracts of mouse skin

The extracts from mouse skin were analyzed by Ouchterlony's agar diffusion method to

Fig. 2. Precipitation pattern of serum components in aged mouse skin obtained with double diffusion plate technique.

Antigens (Peripheral wells):
1: H2O extract
2: 0.15 M NaCl extract
3: 0.45 M NaCl extract
4: 60% EtOH supernatant from 0.45 M NaCl extract
5: Control (normal mouse whole serum)
6: 0.45 M NaCl extract

Antisera (Center wells)
Left side: Rabbit anti-mouse whole serum antiserum
Right side: Rabbit anti-mouse serum albumin antiserum
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Determine whether or not serum components were present in the samples. Antisera against mouse serum and serum albumin were used to test the extracts of H2O, 0.15M NaCl, 0.45M NaCl and 60% EtOH sup fraction of aged mouse skin. Mouse serum was used as the control. As indicated in Fig. 2, the 0.45M NaCl extract and 60% EtOH sup fraction showed no precipitin line either against anti-mouse serum or anti-serum albumin antiserum, while the H2O and 0.15M NaCl extracts and the mouse serum as control presented positive precipitin lines against antimouse serum and antiserum albumin antiserum, though the precipitin line for 0.15M NaCl extracts was very faint.

DISCUSSION

It has been reported that D-Asp was present in metabolically inert protein of long-lived mammals. So far, it has been explained that D-Asp existing in the living organism was derived from racemization of this amino acid during life time at mammalian body temperature. The present experiment showed that D-Asp existed in naturally aged mouse skin and lens in spite of the short life span of this organism. The relation of the D/L ratio of aspartic acid and the rate constant for the racemization reaction at a certain temperature, can be expressed as follows:

\[
\ln \left( \frac{1 + D/L}{1 - D/L} \right) = 2kt + \ln \left( \frac{1 + D/L}{1 - D/L} \right) t = 0
\]

Masters et al.\(^4\) presented a value of 1.67 x 10^{-3} \text{ yr}^{-1} for the rate constant (k), and 0.11 for the time zero (t = 0) term, respectively. These values were obtained by the racemization of aspartic acid in the WI protein of human lens. It is considered that the time zero term in Eq. (1) is due to racemization induced by acid hydrolysis. Mouse body temperature is same as that of humans. If we assume that the k of the human lens protein can be applied to the racemization of the mouse lens protein and that the presence of D-Asp in the WI protein of the mouse lens is due to the racemization process which proceeds according to k, the time required to reach the values of 0.07 or 0.10 for the D/L ratio of aspartic acid would be 9 or 30 years, respectively. Therefore, it seems that the high content of D-Asp in the lens protein of the aged mouse cannot be due to racemization during the animal's life time, because the time required for racemization is much greater than the life span of the mouse.

A higher D/L ratio of aspartic acid was detected in the 60% EtOH sup fraction of 0.45M NaCl extract in skin samples from 4 out of 9 of aged mice. The increase of D-Asp was observed in mice older than at least 20 months, but it did not always occur in all mice after 20 months. One 20-months-old mouse showed a higher D/L ratio of aspartic acid than in older mice (32 months old), as shown in Table 1. The presence or absence of D-Asp may depend on the individual difference after certain age.

The amino acid composition of the 60% EtOH sup fraction in 4 skin samples from the aged mice which contained D-Asp abundantly evidenced that this fraction was not a collagen, a main component of skin, by showing the absence of hydroxyproline and hydroxylysine. Collagen
was detected in the 60% EtOH ppt fraction. From the immunological analysis, it was confirmed that the 60% EtOH sup fraction was different from serum protein. Therefore, it could be considered that the D-Asp rich protein was a minor component derived from connective and/or epidermal tissues. If the presence of D-Asp would be resulted from a racemization during the life time, the D/L ratio of Asp in whole protein from the aged mouse skin should be increased.

The present study indicates that D-Asp accumulated in specific protein(s) in the aged mouse skin and lens. The reason D-Asp is present in the 60% EtOH sup fraction of skin and the lens protein is unknown at the present time. However, the present result suggests that the D-Asp in these samples might be caused in some way other than the above-mentioned racemization during aging.

It has been known that radiation accelerates aging. Our previous study\(^8\) evidenced that the increase of D-Asp in the lens was promoted by gamma-irradiation with 500R. However, it required 18 months after irradiation until an increase of D-Asp was clearly observed. A higher dose of irradiation (1500R) to the head alone induced cataract, but did not increase D-Asp after 3 months. Even if a whole-body irradiation at 37 R per day was continued for 3 months and the total dose reached more than 4000R, an appreciable amount of D-Asp was not detected at 6 months of age. Probably the aging process had not yet been sufficiently promoted in this post-irradiation period. Thus it may be inferred that the increase of D-Asp is not a direct effect of irradiation, as mentioned previously, but an indirect effect of irradiation by promoting the aging process.

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