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Acceleration of Age-related Changes in the Granules of Perivascular Macrophages from the Brain of Senescence Accelerated Mice (SAM)

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Both morphological and quantitative changes in electron-dense granules and foamy granules of the brain perivascular macrophages were investigated using senescence accelerated prone mice (SAMP) at different ages. Perivascular macrophages scavenge high molecular weight substances, such as low-density lipoprotein, incorporating them into granules in the cytoplasm. In this study, SAMP8 and control SAMR1, 2–19 months of age, were examined to characterize these granules in perivascular macrophages of the brain. The number of granules were counted in animals of each age studied and were observed to change with aging from dense to foamy granules in perivascular macrophages. Dense granules gradually diminished starting after 2 months until 10 months postnatally, but foamy granules increased after 4–6 postnatal months. Foamy granules showed a quicker increase in SAMP8 than in SAMR1 around 6 months of age. These results suggested that the morphological changes in granules of perivascular macrophages are characteristics of aging and more accelerated in senescence accelerated SAMP8, than in control SAMR1.

Key words: senescence acceleration, aging, senescence-accelerated mice (SAM), brain perivascular macrophage, foamy cells

Introduction

Perivascular macrophages (fluorescent granular perithelial cells: FGP cells)[1]or Mato's perivascular macrophages[2]are located outside of microvasculatures in Virchow-Robin's spaces of the human's and other mammal's central nervous system (CNS)[1]. According to the expression of murine macrophage subtype markers such as BM8 (MBA, UK)[3] and F4/80[4][5] and rat ED2 (Serotec, UK)[6]~[8], MOMA-2[9], perivascular macrophages in CNS are defined as tissue resident macrophages. There are autofluorescent granules within the macrophages in which scavenged high molecular weight substances are stored[10][11]. They have scavenger receptors in vivo[2][12]~[14] and in vitro[15]. These cells and their granules appear to change morphologically in association with aging in individuals. The granules are associated with lipids such as native or oxidized low-density lipoprotein (LDL) and cholesterol, which are taken up into perivascular macrophages via receptor-mediated endocytosis[16]. Endocytotic vesicles fuse with primary lysosomes in the cells followed by incorporation into electron-dense granules and foamy granules[16]. Therefore perivascular macrophages have been suggested to play a role in promoting atherosclerosis[17][18].

A novel strain of senescence accelerated mice (SAM) was established from AKR/J strain mice at the Department of Senescence Biology, formerly the Department of Pathology, Chest Disease Institute of Kyoto University (Kyoto, Japan), in the early 1970s and was first reported in 1981[19]. In senes-

cence accelerated prone mice (SAMP) the median survival time was 11.9 months and grading score[20] was 6.07 at 8 months of age, which had brief life spans[21] under conventional conditions. In contrast, the median survival time was 17.5 months and grading score was 3.21 at 8 months of age in senescence-resistant mice (SAMR) as a control sub strain, with average life spans[21]. There have been nine senescence-acceleration-prone strains, P1 through P11 (not P4 or P5), termed short-lived SAMP[19)21)22)]. Each SAMP strain exhibits specific pathologic phenotypes such as senile amyloidosis, senile osteoporosis, degenerative joint disease, deficits in learning and memory and so on[21)22)].

In addition to these pathologic phenotypes, the characteristics common to all SAMP are accelerated-senescence after normal development[21]. For instance, they gradually show slow reactivity, passive appearance, glossiness and coarseness in hair, loss of hair, skin ulcers, periophthalmic lesions, corneal opacity, corneal ulcer, cataract and spine lordokyphosis after 6 months postnatally prior to SAMR. It has been proposed that SAMP and SAMR represent a novel murine model for investigating the mechanism that controls the process of senescence[19)21)]. SAMP8 is a sub-strain characterized by learning and memory deficits, as demonstrated by serial behavioral science tests, and also by a low incidence of senile amyloidosis despite the accelerated senescence[21)~23)].

It is important to characterize various aging changes to know the mechanism of senescence. In this study, morphological changes in macrophage granules during aging in both SAMP8 and SAMR1 mice were examined using electron microscopy. All granules were counted and characterized on micrographs. It was demonstrated that the morphological changes in macrophage granules were recognized as one of the characteristics of aging animals and accelerated senescence animals. The acceleration of senescence was observed even in morphological changes of granules in perivascular macrophages from SAMP8.

Materials and Methods

Animals

Male SAMR1 and SAMP8 were used in this study. Animals obtained from the Chest Disease Research Institute of Kyoto University (Kyoto) had been bred in conventional facilities[24]. And after breeding under specific pathogen free (SPF) condition at the Laboratory of Takeda Chemical Ind., Osaka[23)24)], they were raised for 2–3 generations in the laboratory of Oriental Medical Institute (OMI) of Tokyo Women's Medical University under a SPF condition. SAMR1/Kyoto/Ta/OMI, a senescence-resistant strain, and SAMP8/Kyoto/Ta/OMI, a senescence-accelerated strain, were used for this study. Animals were given solid chow (Oriental East Co.,Tokyo Japan) and water *ad libitum*.

In order to check learning and memory abilities, a step-through type passive evasion apparatus consisting of light and dark compartments separated by a guillotine door, was used[23)25)]. When an animal entered the dark compartment of the apparatus, the door was shut afterwards and the mouse was given a foot-shock (0.5 mA) from grids in the floor. The same test was repeated the next day. The mice entered the dark compartment within 300 seconds subjected to the electric shock and were recognized as having learning and memory deficits[23)25)]. These tests were conducted on SAMP8 and SAMR1 at 2 months of age and defective mice were there-by selected. Male SAMR1 and SAMP8, 2 to 19 months of age, were assessed using the same step-through type passive evasion apparatus[23)25)]. While all 2-month-old SAMR1 passed the test, none of the SAMP8 was capable of step-through type passive avoidance. The SAMR1 passed the test and showed no learning and memories deficits, were used as controls for this study.

All of the experimental procedures were carried out according to the principles outlined in the Tokyo Women's Medical University Guide for the Care and Use of Laboratory Animals.

Preparation of specimens for electron microscopy

Animals at each age studied were anesthetized by peritoneal injection of Nembutal (Abbot Lab.

USA) and then perfused with 4% paraformaldehyde and 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) through the left ventricle. Cerebral cortices were dissected, cut into small pieces with razor blades or a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co., UK) and fixed for 2 more hours. After washing with 0.1 M phosphate buffer, tissues were postfixed with 1% osmium in 0.1 M phosphate buffer (pH 7.4) for 2 hours. The tissue blocks were dehydrated through graded series of ethanol and embedded in epoxy resin after soaking in propylene oxide. Embedded tissue blocks were cut into thin sections. These sections were stained with uranyl acetate and lead citrate. Cerebral cortical perivascular macrophages were observed by electron microscopy (Hitachi H7000, Hitachi, Japan).

Counting granules in perivascular macrophages

In thin sections from animals of different ages (total 106 random cell sections), electron dense granules were counted. Since fused foamy granules were difficult to visualize separately for individual counting, the foamy granules were determined by counting vacuoles. The ratios of dense and foamy granules to total granule numbers are shown as percentages separately for each perivascular macrophage examined. The relations between granule numbers and aging were assessed in 3–5 animals of each age group.

Statistic study

The significant prognostic variables were identified using general linear models. The p-value of less than 0.05 was regarded as indicating a statistically significant difference. The statistical analysis was performed using the general linear model (GLM) procedure and statistic analysis system (SAS), version 8.2 (SAS Institute, Cary, NC. USA). The relation between the granules and ages was investigated by Pearson's correlation coefficient test and linear regression. The sign of the correlation in the foamy granules retained from that of the regression coefficient.

Results

Morphology of perivascular macrophages in SAMR1 and SAMP8

Perivascular macrophages were located in Virchow-Robin's space surrounded by the basal lamina of endothelial or smooth muscle cells, and a glial limiting membrane (Fig. 1a). Nuclei were irregularly shaped and rich in chromatin. Small vesicles, rough endoplasmic reticulum, mitochondria, Golgi apparatus, and high electron density granules were seen in the cytoplasm. Plasma membranes showed deep infolding into the cytoplasm, and plasmalemmal infolding was often observed at sites of endocytosis.

Round granules containing highly electron-dense materials, with diameters of 0.1–1 μm, were seen in 2 month-old SAMR1 and SAMP8 (Figs. 1a, 1b). Foamy granules with small vacuoles were often observed in perivascular macrophages from SAMP8 at 3 months of age, but only rarely in SAMR1 of the same age (not shown).

In 7 month-old SAMR1 and SAMP8 (Figs. 2a, 2b), there were numerous granules in the cytoplasm of perivascular macrophages. Endocytotic vesicles were also observed in the cytoplasm. Electron-lucent granules in the cytoplasm consisted of much smaller electron-lucent foam-like vacuoles and were considered to be foamy granules (Figs. 2a, 2b). The foamy granules were more abundant in 7 month-old SAMR1 and SAMP8 (Figs. 2a, 2b) than in 2-month-old SAMR1 and SAMP8 (Figs. 1a, 1b).

In SAMR1 at 12 months of age (Fig. 3a), perivascular macrophages contained numerous cytoplasmic granules and showed swelling on the luminal surface of blood vessels often at vessel bifurcations. Endocytotic vesicles, rough endoplasmic reticulum, mitochondria and Golgi apparatus were sparse in the cytoplasm (Figs. 3a, 3b). Foamy granules were filled with small electron-lucent vacuoles (Figs. 3a, 3b) and large electron-denser granules were observed (Figs. 3a, 3b). Only one new young type macrophage with small dense granules was found in older mice (Fig. 3b). More foamy granules and fewer dense granules were seen in perivascular macrophages from 19-month-old SAMP8 (Fig. 4).

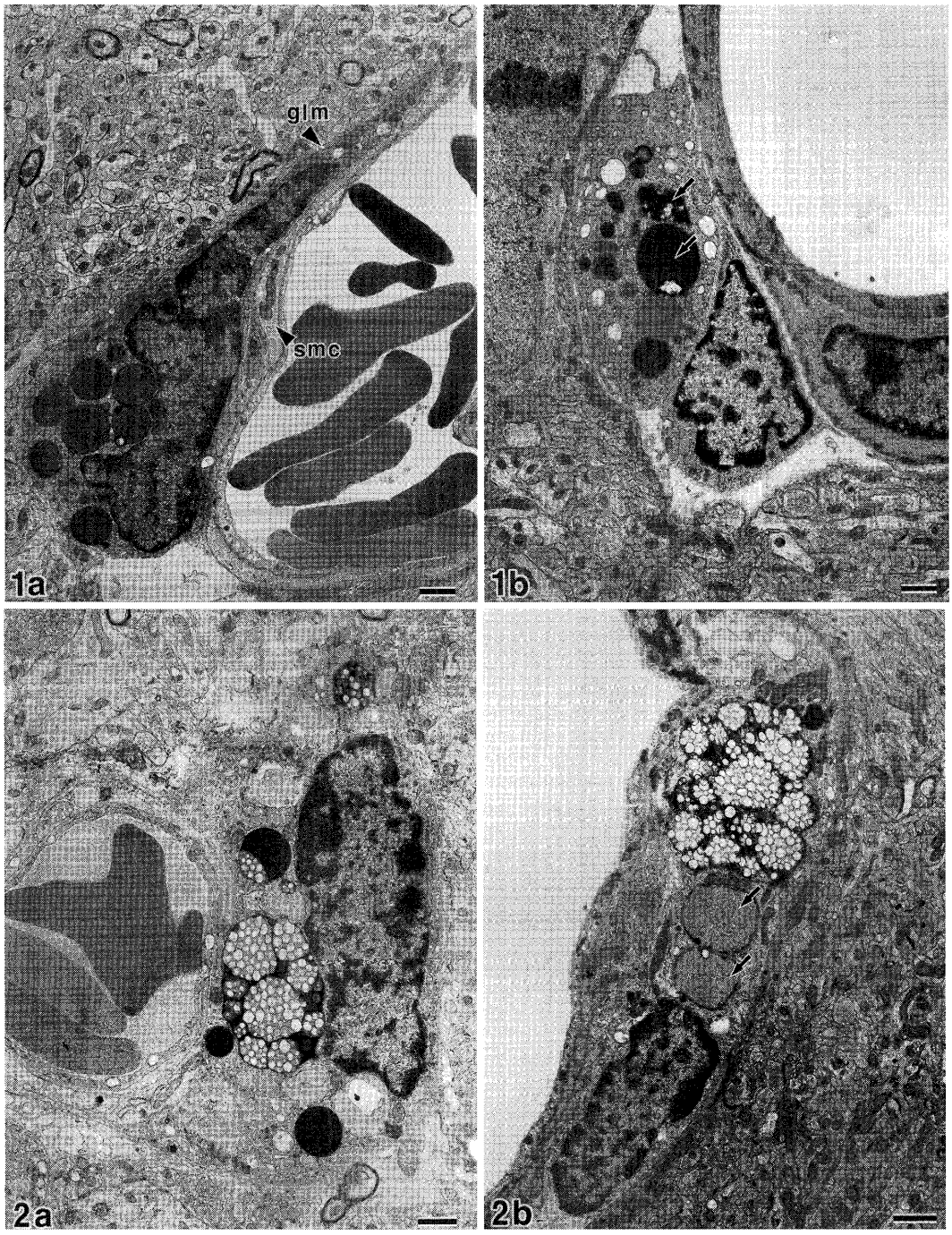


Fig. 1 Perivascular macrophages and blood vessels in longitudinal sections from 2-month-old SAMR1 and SAMP8

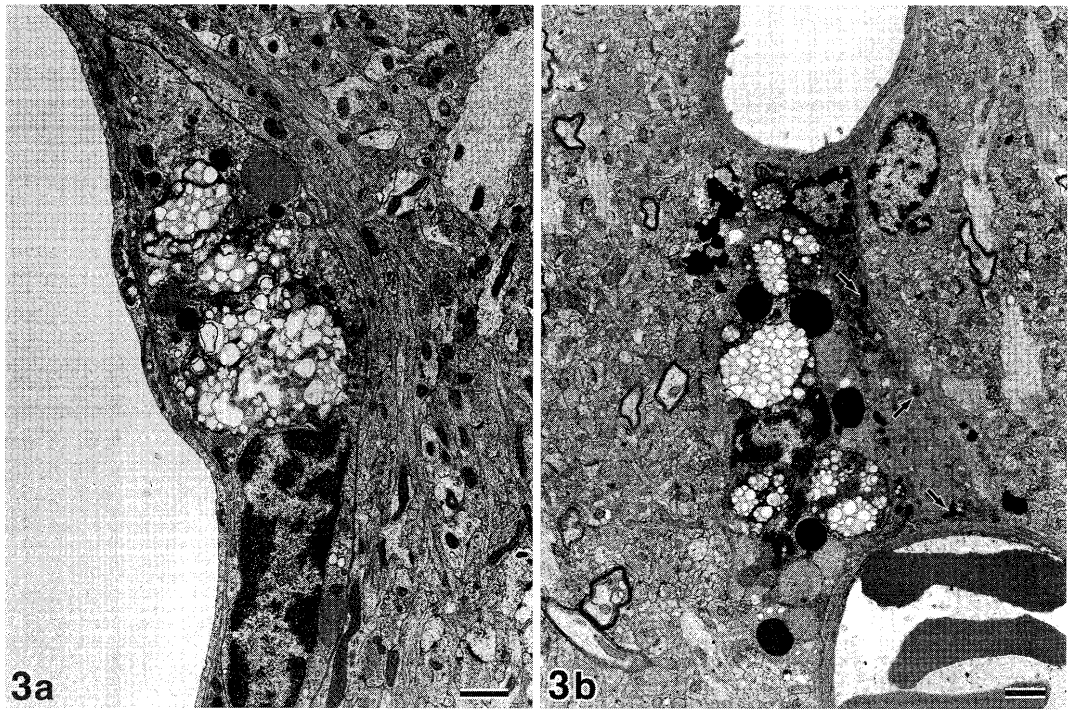
a: A perivascular macrophage present between the basal lamina of a smooth muscle cell and the glial limiting membrane in the space of Virchow-Robin of a SAMR1. Round and electron dense granules (granules in the primary stage) are seen in the cytoplasm. Arrow heads with smc or with glm show smooth muscle cell and glial limiting membrane, respectively.

b: A perivascular macrophage from a SAMP8. Dense granules (arrows) including small amounts of foam can be seen in the cytoplasm of a perivascular macrophage. Bar: 1 μm

Fig. 2 Perivascular macrophages from SAMR1 and SAMP8 at 7 months of age

a: A perivascular macrophage from a 7-month-old SAMR1 in cross section. Electron lucent and relatively small vacuoles (foams) can be seen in granules.

b: A perivascular macrophage from a 7-month-old SAMP8 in longitudinal section. In addi in the perivascular macrophages. Bar: 1 μm..



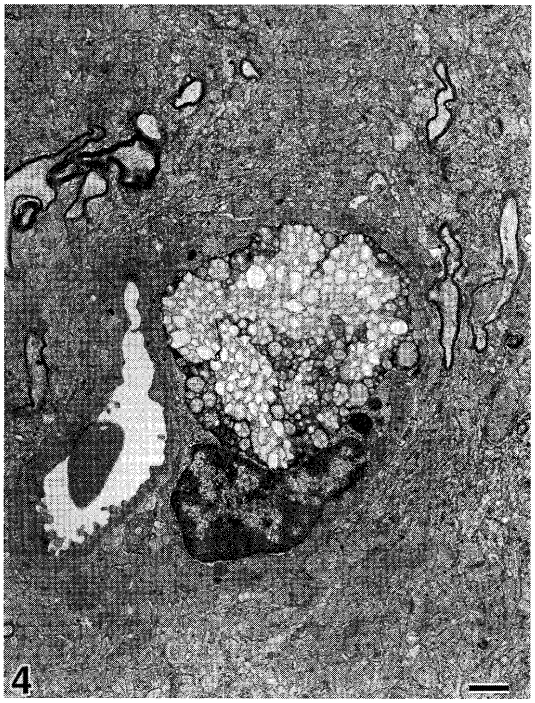


Fig. 3 Perivascular macrophages from 12-month-old SAMR1 and SAMP8
a: A perivascular macrophage from a 12-month-old SAMR1 in longitudinal section of a microvessel. Large foamy granules can be seen in the perivascular macrophage.
b: Perivascular macrophages from 12-month-old SAMP8 in a cross section. Granules including relatively small vacuoles (foam) and electron-opaque granules can be seen. These granules occupy most of the cytoplasm of swollen cells. Another macrophage looking relatively young, with only small dense cytoplasmic granules (arrows) is also seen. Bar: 1 μm

Fig. 4 A vessel in a cross section containing a perivascular macrophage, from a 19-month-old SAMP8 Numerous foamy granules occupy the cytoplasm of a swollen perivascular macrophage. Bar: 1 μm..

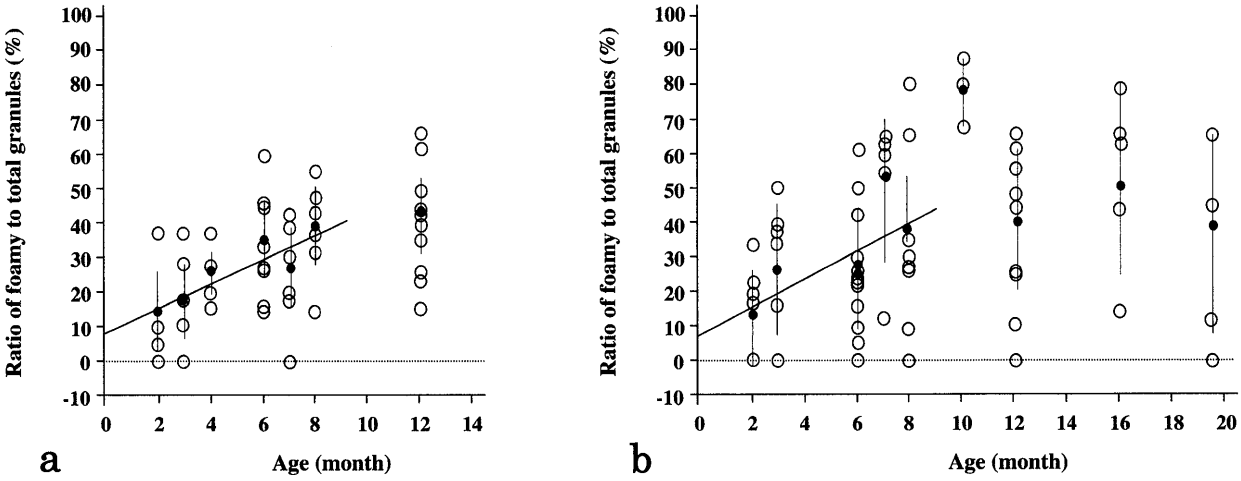


Fig. 5 The relation between foamy granules and aging
The ratio of foamy to total granules is shown on the Y-axis and ages of the animals are shown on the X-axis. The foamy granules gradually increased in number with age. The increase in foamy granules was greater in SAMP8 (b) than SAMR1 (a) mice. Closed circles (●) show mean values of the ratio of foamy granules. The increase of foamy granules with ages is shown in the columns of SAMR1 (a) and SAMP8 (b) as regression lines separately. The increase of foamy granules in SAMP8 was greater and faster than those in SAMR1.

In addition to dense granules and foamy granules, moderately electron dense granules were observed in perivascular macrophages from 2-, 7- and 12-month-old animals (Figs. 1b, 2b, 3a and 3b).

Changes in numbers of granules; relationships between dense and foamy granules with age

Round electron-dense granules (Figs. 1a, 1b) have often been observed in perivascular macrophages from SAMR1 and SAMP8 at 2 months of age. Dense granules with small vacuoles appeared in the cytoplasm and gradually became foamy granules via the vacuolation. Most granules in the perivascular macrophages of younger mice were dense (Figs. 1a, 1b), apparently due to the ingestion of lipids such as apo-lipoproteins. While the dense granules decreased with age, foamy granules and the foam within them increased with age in both SAMR1 (Fig. 5a) and SAMP8 (Fig. 5b). The amount of foams increased in SAMP8 more quickly than in SAMR1 at 7 months of age (Figs. 2a, 2b, 5). The regression lines were shown as $y = 3.5x + 7.9$ in SAMR1 (Fig. 5a) and $y = 4.0x + 7.5$ in SAMP8 (Fig. 5b) of 2 months until 8 months postnatal animals from regression coefficients respectively.

The results of the variance analysis were shown in Table. The p-value which gave $Pr > F$, were 0.0132 in SAMR1 and 0.0006 in SAMP8, respectively. Therefore, variance in SAMR1 and SAMP8 were highly significant. The ages (months) and the amount of vacuoles in foamy granules have revealed correlations from 2 months until 8 months postnatal in SAMR1 ($|R| = 0.475$) or P8 mice ($|R| = 0.400$), where R represents the correlation coefficient.

Discussion

Morphological changes in granules of perivascular macrophages were examined in this study. The number of granules changed with age from dense to foamy granules in perivascular macrophages. Dense granules gradually diminished starting after 2 months until 10 months postnatally, but foamy granules increased after 4-6 postnatal months. These phenomena were considered to reflect the aging of animals, according to the results of statistical analysis and the observation in an electron microscopy (Figs. 1-4). The moderately electron dense granules seem to be different from dense granules and foamy granules[16] and not to relate to the foamy granule formation from the report[16].

Perivascular macrophages apparently did not

Table Variance analysis in SAMR1 and SAMP8

Dependent variable: source	DF	Sum of squares	Mean square	F values	p
SAMR1					
Model	6	4,217	702	3.15	0.0132
Error	38	8,478	223		
SAMP8					
Model	8	15,273	1,909	4.17	0.0006
Error	54	24,696	457		

The significant prognostic variables were identified using general linear models and were revealed to be statistically significant in both SAMR1 and SAMP8 strains.
DF: degree of freedom, p: probabillity of Pr > F (Pr: prognostic variables, F: Fisher's values)

multiply after the 2nd postnatal month when they were capable of ingesting substances. The distribution of ink-labeled perivascular macrophages from rats did not change markedly after 2 years suggesting high stability of this cell pool in rats[11]. Macrophages supervitally labeled with fluorescent- and rhodamine-conjugated dextran amines, remained stable for at least 8 weeks[26], and only 6.4% of perivascular macrophages in Virchow-Robin's space were new arrivals during the prior 4 weeks[26] in rats. Cell division appears[27] to be very rare after the capacity to scavenge is obtained[11)26]. Because the populations of young macrophages were minor, it appeared that scavenging macrophages in Virchow-Robin's space had a very slow turnover. Slow turnover of perivascular cells in normal animals, was reported by several investigators[28)~34)] but not in SAM. The number of dense granules decreased with age, in parallel with the change to foamy granules, but these granules never disappeared entirely in this study (Fig. 4). This phenomenon indicated that newly arriving young macrophages with cytoplasmic dense granules coexist with the older macrophages (Fig. 3b).

Senescence was accelerated in SAMP8 as compared to SAMR1. The formation of foamy granules was also accelerated in perivascular macrophages of SAMP8 as compared to those of SAMR1 until 8 months after birth (4.0 in SAMP8 and 3.5 in SAMR1). The formation is led by oxidative modification of LDL in macrophages[35]. It is reported that perivascular macrophages took up modified lipoproteins and stored them as cholesteryl ester in foamy granules after digestion[16)17)]. The one modification of LDL *in vivo* may result from an interaction with malondialdehyde, is released from blood platelets or is produced by lipid peroxidation at the site of arterial injury[17]. Nomura et al[36] reported that the content of malondialdehyde was significantly higher in the liver and brain of 11–12-month-old SAMP8 than in SAMR1. The SAMP8 liver also showed less activity of superoxide dismutase than in SAMR1. From these reports, the brain and liver of the SAMP8 in 11–12-month-old seem to fit easier circumstances than SAMR1's in the cholesteryl ester accumulation. Higuchi et al[37] reported the serum LDL levels in SAMR1, R2 and SAMP1, P2. There has not been, however, direct comparative data on the levels of LDL in SAMP8 and SAMR1[37].

The number of lysosomal granules in perivascular macrophages was also related to the ages of the rats[38]. β-Galactosidase is a lysosomal enzyme which functions at pH 6.0 or 4.0. The activities of this enzyme at pH 4.0 were detected in lysosomal granules of the perivascular macrophage of rats[38]. The reactive granules corresponded to lysosomes showing acid phosphatase (ACP-ase) activity in perivascular macrophages. With aging ACP-ase activity was increasingly activated and the uptakes of various substances also increased in perivascular macrophages. The data in SAM about β-galactosidase staining are not found.

Learning and memory deficits among observed senile changes are either with or without brain atrophy[21)24)]. The source of these deficits involves the process of attainment of memory construction in

SAMP8, while the memories were normally maintained[39]. In certain parts of the brain in SAMP8, PAS positive granular structures were observed[40)41)], and cathepsins E and D were increased in reactive microglial cells[42] associated with spongiform degeneration[39)43)] in the brain stems of SAM. Even in 2-month-old SAMP8 learning and memory deficits were already apparent, indicating that these deficits might be more fundamental, and even involve the process of memory construction[39].

The possible mechanism of dysfunction of learning and memory in the brain of SAMP8 was shown in the previous report[44]. The deficit starts as early as 2 weeks after birth in this strain. The relation between morphological changes in perivascular macrophages and dysfunction of learning and memory in the brain of SAMP8 was not clarified in this study. Among these obtained strains the one shows learning and memory deficits in passive avoidance responses even at 2 months of age was designated as SAMP 8. Animals used in this study were checked their learning and memory deficits. This selection effects might be reflected to the p-values in Table.

In conclusion, various phenomena related to accelerated senescence have been observed in SAM. The morphological changes in perivascular macrophages were suggested to be among these phenomena associated with senescence. The formation of foamy granules in SAMP8 was accelerated as compared that in SAMR1. Greater acceleration of senescence was seen in SAMP8, as reflected by the granule changes in perivascular macrophages from this strain.

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老化促進マウス（SAM）の血管周囲マクロファージ内顆粒における加齢変化の促進

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老化促進マウス（SAMP）の加齢変化は，対照群となるSAMRよりも早く現れることが知られている．著者らは，これまでラット脳血管周囲マクロファージが加齢とともに，その数や細胞形態において変化することを報告してきた．そこで，この研究では，SAMP8と対照群のSAMR1を用いて，脳の血管周囲マクロファージの電子密度の高い顆粒（dense granule）と泡沫状顆粒（foamy granule）について，それぞれの形態と数の変化について年齢を追って観察した．顆粒は加齢とともにelectron-denseからfoamyに変わりdense granuleは減少する．逆にfoamy granuleは4〜6ヵ月頃から急速に増え，さらに加齢とともに増える．以上の結果より，マクロファージ内のfoamy granuleへの変化は，老化の初期段階の兆候の一つであり，この兆候は，SAMP8においてSAMR1より，促進されていた．