Acta Neuropathol (Berl) (1986) 72:124–129

Acta Neuropathologica

Periodic acid-Schiff (PAS)-positive, granular structures increase in the brain of senescence accelerated mouse (SAM)*

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Summary. Abnormal granular structures, which stained positively with periodic acid-Schiff (PAS-positive granular structures; PGS), were observed in the brain of senescence accelerated mouse (SAM). They were small, round to ovoid, homogenous structures measuring up to 5 μm in diameter and usually grouped in clusters. PGS were localized in the hippocampus, piriform cortices, olfactory tubercle, nucleus of the trapezoid body, and cerebellar cortices. Quantitative analysis revealed that PGS remarkably increased in the hippocampus of SAM-P/8, a substrain of SAM, with advancing age, although a few PGS also appeared in the aged control mice, SAM-R/1 and DDD. Their histochemical nature, morphological features and distribution pattern were different from those of corpora amylacea and other similar bodies. A close anatomical relationship between PGS and glial fibrillary acidic protein-positive astrocytes was inferred from immunohistochemical studies. PGS is considered to be one of the morphological manifestations of senescence in mice brains, and are found to occur more numerously in the brains of learning or memory deficit mice, SAM-P/8.

Key words: Aging – PAS-positive granular structures – Learning disturbance – Senescence accelerated mouse (SAM)

Senescence accelerated mouse (SAM), a murine model of accelerated senescence, was established by Takeda and his colleagues (Takeda et al. 1981; Hosokawa et al. 1984a). Accelerated senescence prone mouse (SAM-P) consists of SAM-P/1, SAM-P/2, SAM-P/3, SAM-P/4, SAM-P/6, and SAM-P/8, and accelerated senescence resistant mouse (SAM-R) consists of SAM-R/1, SAM-R/2, SAM-R/3. SAM-P shows earlier onset and irreversible advancement of senescence revealed by analysis of aging dynamics such as Gompertz function, survivorship curve and grading score system. Systemic senile amyloidosis (Takeshita et al. 1982; Higuchi et al. 1983), cataract (Hosokawa et al. 1984b) and osteoporosis (Matsushita et al. 1986) were also reported as characteristics and age-related pathologies of these mice.

Recently SAM-P/8/Ta, SAM-P/8 bred under specific pathogen-free conditions, was found to show an age-related deterioration of learning abilities (Miyamoto et al. 1986). SAM-P/8/Ta exhibited a significant avoidance deficit in the passive avoidance task, and prolonged performance time in the multiple-T water-maze task compared with control mice, SAM-R/1/Ta. Both deteriorations were accelerated with advancing age. Because no significant difference of shock sensitivity or mobility in the maze performance was observed between these two mice substrains, they concluded that these behavioral abnormalities were ascribed to learning or memory deficits of SAM-P/8/Ta.

During an attempt to find a structural basis for such behavioral changes, we found that an abnormal structure, which stained positively with periodic acid-Schiff (PAS), increased with aging in the brain of SAM-P/8. Similar structures were also observed in aged control mice, although they were extremely few. The purpose of this study is to describe the light microscope features, histochemical nature, topographical distributions, and age dependency of those abnormal structures. This is the first report of these abnormal structures, and their numerous occurrence in the brain of memory-deficit mice should be considered significant.

* Supported by grants from the Japanese Ministry of Education, Culture and Science and the Ministry of Health and Welfare
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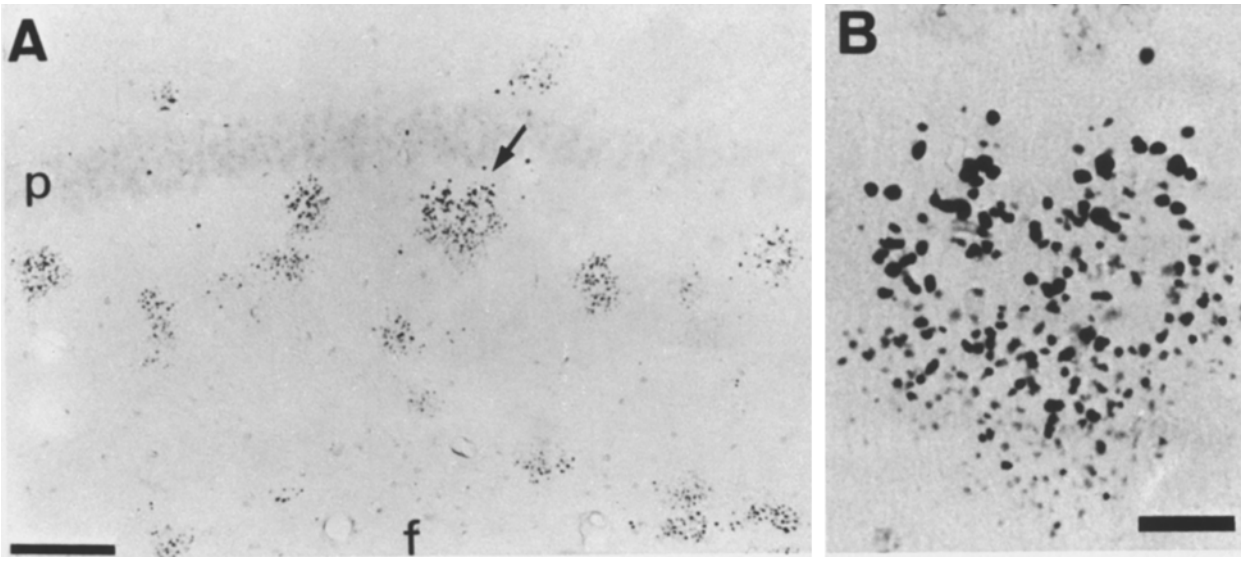

Fig. 1. A Coronal section of hippocampus from a 16-month-old SAM-P/8. Many clusters of PAS-positive granular structures (PGS) are observed. *p*: pyramidal cell layer; *f*: hippocampal fissure. Bar = 100 μm. **B** Enlargement of one cluster of PGS in **A** (*arrow*). Bar = 20 μm. Blumer's PAS-dimedone, lightly counterstained with cresyl violet

Methods

SAM-P/8, SAM-R/1 and DDD mice were used in this study. Both male and female were included. The mice were reared in our laboratory under conventional conditions at 24° ± 2° C and were maintained on a commercial diet (CE-2, Nihon CLEA) and tap water ad libitum.

Twenty-four SAM-P/8, 14 SAM-R/1 and 14 DDD mice were killed by deep anesthesia with diethylether. The brains were quickly removed, fixed in 10% neutral formalin, and embedded in paraffin. Six-micrometer frontal sections were cut serially, and every tenth section including hippocampus was stained with PAS and used for quantitative analysis. Other sections were stained with hematoxylin and eosin (H.E.), Kluever-Barrera (K.B.) and Bodian's silver impregnation routinely. Blumer's PAS-dimedone (Blumer 1959), Schiff's, Alcian blue (pH 1.0 and 2.5), toluidine blue (pH 1.5, 3.0 and 4.5), Alkaline Congo red (Puchtler et al. 1962), Millon's and ninhydrin-Schiff were also employed. Enzyme digestions were performed with α-amylase (Sigma, USA; 100 units/ml in 0.02 M phosphate buffer, pH 6.8) and β-amylase (Sigma, USA; 150 units/ml in 0.016 M acetate buffer, pH 4.8) for 2 to 24 h at 37° C. Frozen sections were obtained from 12-month-old SAM-P/8 and stained with iodine, Sudan black B, oil red O and Okamoto's α-naphthol for glycolipids (Okamoto et al. 1948). Unstained sections were also examined under fluorescence microscopy.

Five aged SAM-P/8 (10 – 13 months of age) were perfused transcardially with phosphate-buffered saline, pH 7.4, followed by phosphate-buffered 4% paraformaldehyde (FA), 0.3% glutaraldehyde (GA) and 0.2% picric acid (PA), pH 7.4. The brains were immediately removed and postfixed phosphate-buffered 4% FA, 0.2% PA, pH 7.4. Following cryoprotection in phosphate-buffered 15% sucrose, 20-μm thick frontal sections were cut serially with a cryostat. The first series of every fifth section was stained with PAS and used for the mapping of the abnormal structure. Other sections were washed with phosphate-buffered saline containing 0.3% Triton X-100, and sections including hippocampus were selected to be processed for glial fibrillary acidic protein (GFA) immunocytochemical study following the PAP technique (Sternberger 1979) with some modifications (Kimura et al. 1981). Sections were incubated serially in rabbit anti-human GFA (DAKO, Denmark; 1/20,000) (Onteniente et al. 1983), in goat's anti-rabbit immunoglobulins (Miles-Yeda, Israel; 1/5,000) and in peroxidase-rabbit's anti-peroxidase complex (PAP) (DAKO; 1/1,000). Following visualization of peroxidase labeling, sections were mounted on glass slides and counterstained with PAS.

Results

The abnormal PAS-positive granular structures (PGS), viewed with a light microscope, were small, round to ovoid, homogeneous structures measuring up to 5 μm in diameter (Fig. 1 B). They were frequently grouped in clusters. A large cluster often consisted of several tens of PGS and ranged in size up to 50 μm Fig. 1 A, B).

The histochemical results are summarized in Table 1. PGS did not stain with H.E., K.B., Alkaline Congo red, Alcian blue or toluidine blue. Silver impregnation using Bodian's method showed weak argyrophilia. None stained with Sudan black B, oil red O or Okamoto's α-naphthol for lipid or glycolipid. Millon's and ninhydrin-Schiff for protein were also negative. Unlike lipofuscin, PGS did not show an autofluorescence under fluorescence microscopy. Schiff's reaction without periodic acid oxidation was negative. An incubation in the saturated dimedone solution for 30 min at 60° C after periodic acid oxidation did not block the red Schiff staining of PGS. The staining intensity with PAS was slightly reduced after

Table 1. Histochemical properties of PAS-positive granular structures (PGS) in the hippocampus from aged SAM-P/8

Method	Result
Hematoxylin and eosin	−
Klüver-Barrera	−
Bodian's	+ weak
Congo red	−
Alcian blue	−
Toluidine blue	−
Autofluorescence	−
Sudan black B	−
Oil red O	−
Okamoto's α-naphthol	−
Millon's	−
Ninhydrin-Schiff	−
Schiff's	−
Periodic acid-Schiff	+
Blumer's PAS-dimedone	+
α-Amylase/PAS	Intensity ↘
β-Amylase/PAS	no change
Iodine	+ weak, lavender

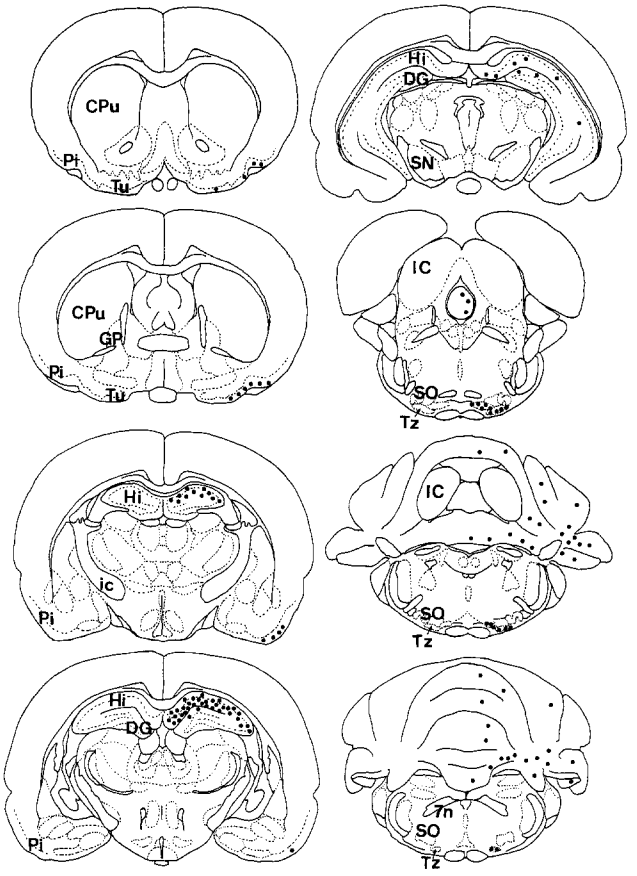

Fig. 2. Distribution of PGS in the brain of SAM-P/8. One cluster of PGS is represented by a *solid dot* in the right hemisphere. *CPu*: caudate putamen; *Pi*: piriform cortex; *Tu*: olfactory tubercle; *GP*: globus pallidus; *Hi*: hippocampus; *ic*: internal capsule; *DG*: dentate gyrus; *SN*: substantia nigra; *IC*: inferior colliculus; *SO*: nucleus of superior olive; *Tz*: nucleus of trapezoid body; *7n*: facial nerve

long incubation with α-amylase, but β-amylase did not appear to be effective. PGS, however, were far more resistant to α-amylase digestion than muscle and liver glycogen, which completely disappeared after 4 h incubation. PGS did not disappear after more than 24 h incubation in this enzyme solution. Iodine reaction showed weak but unequivocally positive staining of a lavender color.

The topographical distribution of PGS in the brain of SAM-P/8 is shown in Fig. 2. PGS were most frequently found in the hippocampus, especially in CA1, CA2 and CA3. Although they were disseminated in all of the hippocampal layers except alveus, the most prominent distribution was observed in the stratum radiatum. Some PGS were seen in the molecular layer of the dentate gyrus. A number of PGS were also found in the molecular layer of the olfactory tubercle and piriform cortices. In the posterior part of the brain, most were localized in the nucleus of the trapezoid body and cerebellar cortices. In the cerebellar cortices, the Purkinje cell layer and the granular layer were mainly affected. The morphological features of PGS were similar in all parts of the brain. In the cerebellar cortices, however, each cluster tended to consist of a fewer number of PGS, and in the nucleus of the trapezoid body the borders of the clusters were sometimes unclear.

For quantitative analysis, six level-matched sections were selected and the number of the clusters of PGS in the bilateral hippocampal regions was counted. Small clusters which consisted of less than five PGS were neglected. Mice of both sexes were examined, and no difference between males and females was observed. SAM-P/8 of more than 16 months old were not available because they rarely survived up to that age. The mean life span of SAM-P/8 was about 9 months and that of SAM-R/1, about 13 months (Takeda et al. 1981), as was DDD. Figure 3 shows the age-dependent increase of PGS in the brains of these mice. In SAM-P/8, PGS appeared in the hippocampus at 3 months old, and a number of them increased rapidly with aging from 6 months old. PGS were also observed in the control mice, SAM-R/1 and DDD, into old age. Even if the difference of mean life span was taken into consideration, however, the occurrence of PGS in the control mice was much later and the increment with aging was much less than in SAM-P/8.

One of the morphological characteristics of PGS was their close relationship to astrocytes observed in the double-stained sections of hippocampus with GFA

immunocytochemistry and PAS. A GFA-positive astrocyte was often situated in the center of a cluster of PGS (Fig. 4A), and each PGS appeared to exist in contact with the processes of that astrocyte (Fig. 4B). Commonly, only one astrocyte seemed to relate to one cluster of PGS, and two or more astrocytes extending their processes to the same cluster of PGS was not observed. There were also a number of PGS clusters, however, which did not accompany GFA-positive astrocytes.

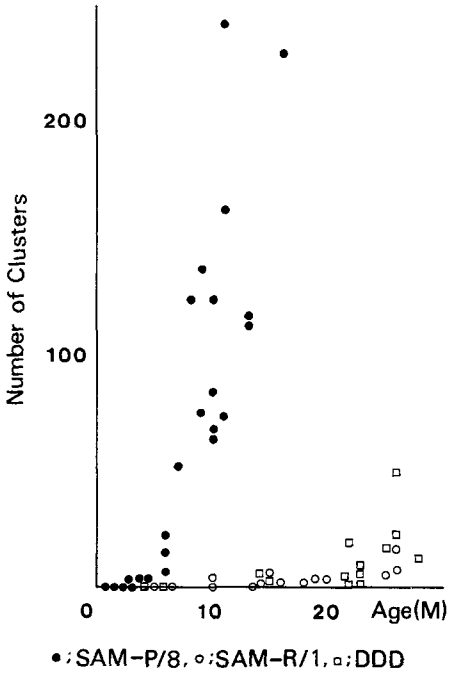

Fig. 3. Number of clusters of PGS in the hippocampus of six level-matched 6-μm-thick sections

Discussion

PGS increase markedly with aging in the brain of SAM-P/8. On the basis of the histochemical nature of PGS, they are suspected to contain certain amounts of glucose polymers as a part of their polysaccharide molecules. Both α- and β-amylase act on α-1,4 glucosidic linkages of glucose polymer. α-Amylase splits the linkages of polysaccharide molecule in an apparently random fashion, whereas β-amylase attacks polysaccharide chains, effecting successive removals of maltose units from the non-reducing ends. Therefore, it is likely that PGS contain α-1,4 glucosidic linkages, but other linkages may also exist in the molecule. Glycogen, consisting of glucose polymer with α-1,4 and α-1,6 glucosidic linkages, is much more susceptible to α-amylase than PGS. However, when glycogen is highly aggregated or is tightly bound to proteins in the granule, it may become more resistant to amylolytic enzymes (Sakai et al. 1969). Blumer's PAS-dimedone method is considered to be more specific than the conventional PAS technique. Dimedone reacts with the aldehydes formed by periodic acid oxidation of polysaccharides, and after

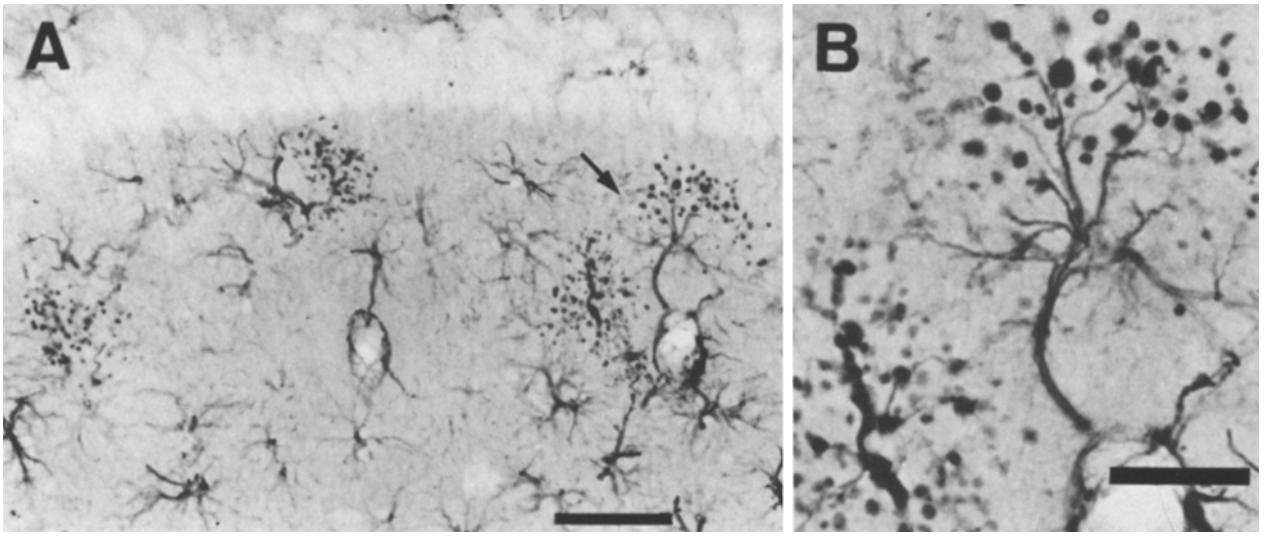

Fig. 4. **A** Coronal section of hippocampus from a 13-month-old SAM-P/8. Several clusters of PGS are present and a glial fibrillary acidic protein (GFA)-positive astrocyte is observed in each cluster of PGS. Bar = 50 μm. **B** Enlargement of two clusters of PGS with astrocytes in **A** (*arrow*). Each PGS seems to be situated along the process of the astrocyte. Bar = 20 μm. Double staining with GFA immunocytochemistry and PAS

dimedone treatment, these aldehydes can no longer stain red with Schiff's reagent. Dimedone, however, does not block the red Schiff staining of highly polymerized hexoses such as glycogen under the standard conditions selected (Blumer 1959; Sakai et al. 1969). The PAS reaction of PGS was apparently undiminished after dimedone treatment under the same condition. The lavender color with iodine staining suggests that PGS contain long linear chains of glucose polymers. It should be noted that glycogen, with branched chains whose linear portions are relatively short, stains a brown color with iodine. Other histochemical results show that PGS do not contain much protein or lipid. In addition, the staining properties of PGS do not resemble those of lipofuscin, amyloid or acid mucopolysaccharides.

PGS were only found in some regions of the brain, and their distribution pattern was unique. They were localized in the allocortices such as the hippocampus, piriform cortices and olfactory tubercle in the forebrain, and were not found in the neocortices. The selective anatomical distributions of PGS may be related to the cause of the formation of these structures. How PGS are formed in the aged mouse brain remains unknown, but their close relationship to GFA-positive astrocytes observed in the hippocampus suggests that these astrocytes may play an important role in the process of the formation of PGS.

To our knowledge, no similar structure has been observed in any other animal species including human beings. Corpora amylacea are round PAS-positive intracytoplasmic bodies which usually distend the processes of fibrous astrocytes (Ramsay 1965). They occur in certain pathological conditions and are particularly common in the senile brain. Similar bodies were observed in some animal species (Suzuki et al. 1978, 1979). Lafora body-like structures in the cerebellar cortices were reported in an electron microscope study of aged ICR mice, but its light microscope feature was not mentioned (Nakamura et al. 1973). However, corpora amylacea and similar bodies, unlike PGS, stain positively with H.E. or Alcian blue, and many of them have a darker basophilic core (Ramsay 1965). Moreover, they are not known to form clusters. Our observations with H.E. staining failed to find corpora amylacea-like structures in the brain of SAM-P/8.

In human brains many age-associated morphological changes are known, among which much attention has been paid to senile plaques and Alzheimer's neurofibrillary tangles, because they appear far more frequently in the brain of Alzheimer's disease and senile dementia of Alzheimer's type. On the other hand, only a few morphological parameters of aging are available in the brain of mice which are used in many experimental investigations of senescence. Neuroaxonal dystrophies and eosinophilic thalamic intraneuronal inclusions (Fraser 1969) are known to increase in the aged mouse brain. Lipofuscin accumulation is a common pathology correlated with aging (Nandy 1966), but no significant difference between SAM-P/8 and SAM-R/1 could be detected by a morphometric study about this age pigment (unpublished data).

The results of this study revealed an age-associated increase of PGS in the brain of both SAM-P/8 and the control mice, SAM-R/1 and DDD. It should be emphasized that PGS is considered to be a new morphological manifestation of senescence, and that they appear more numerously in the brains of learning or memory deficit mice, SAM-P/8.

Further study will be required to determine whether a correlative link exists between the unusual increase of PGS and the behavioral abnormalities which SAM-P/8 show with advancing age.

Acknowledgements. Gratitude is extended to Dr. Yoshihiro Ishihara (Department of Pathology, Tokyo Metropolitan Institute for Neurosciences) for pertinent advice. The authors would also like to thank Mr. Takatoshi Matsushita and Miss Yoshimi Kitasaka for technical assistance.

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Received July 1, 1986/Accepted July 14, 1986