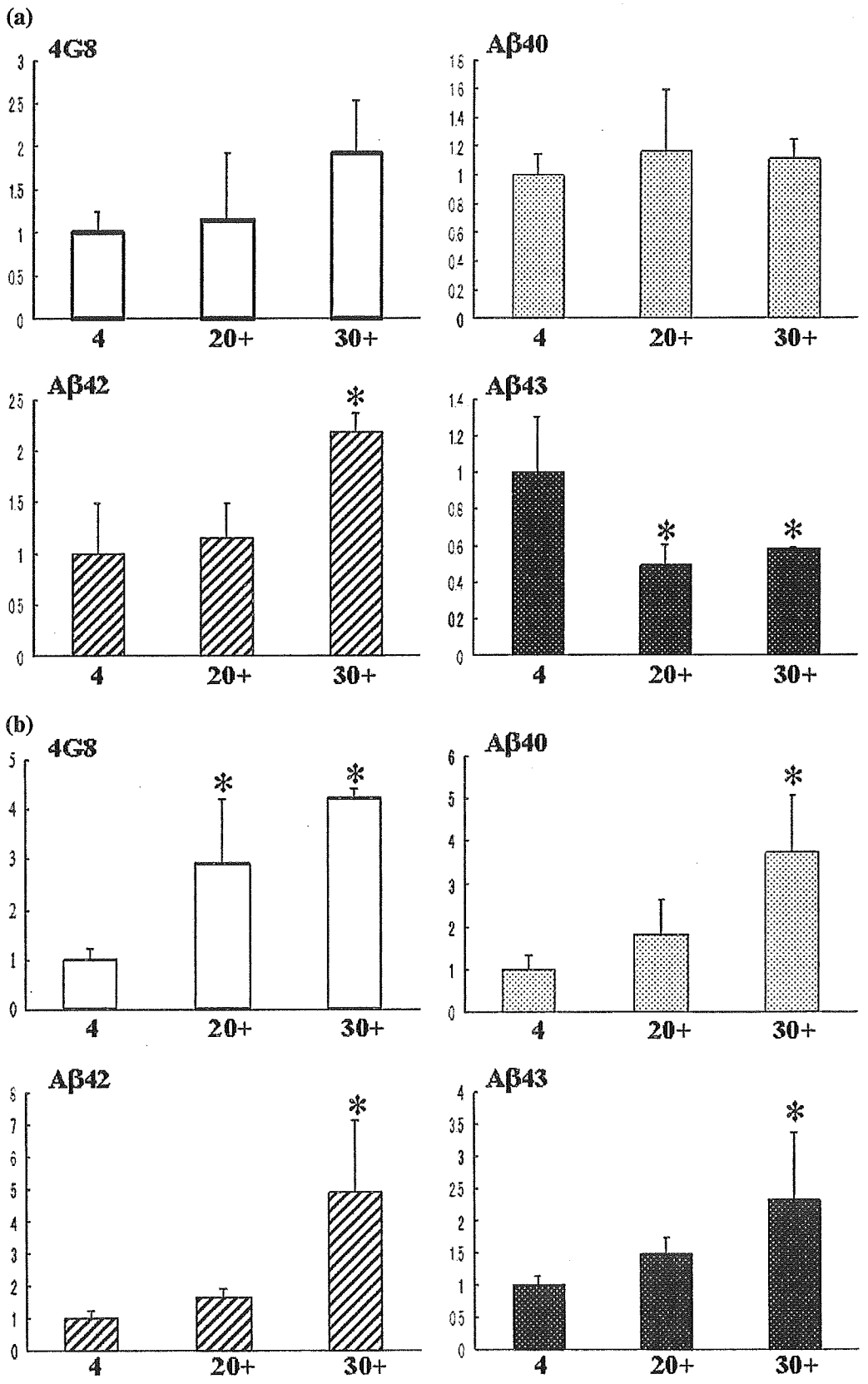
(a)
4G8
Aβ40
Aβ42
Aβ43
(b)
4G8
Aβ40
Aβ42
Aβ43
4
20+
30+

Discussion

In the present study, immunohistochemical and biochemical methods were used to investigate the localization and age-related changes of intracellular Aβ in brains from young, adult and aged cynomolgus monkeys.

Recent studies have shown that intracellular Aβ is found only in the brains of aged individuals or brains of AD patients, but not in brains of nonhuman primates [9,10,12,16]. However, in the present study, we found intracellular Aβ in cortical neurones of monkeys of various ages (Figures 1 and 2). One possible technical explanation for this apparent discrepancy is that in previous studies, material came from brains fixed with neutral buffered formalin, whereas in the present study material was derived from brains fixed with paraformaldehyde [9,10,12,16]. For immunohistochemical studies we fixed brain samples, derived from the same monkeys, with either neutral buffered formalin or paraformaldehyde. Anti-Aβ antibodies failed to immunostain intracellular Aβ in cortical neurones from samples fixed with the formalin protocol (data not shown). On the other hand, all anti-Aβ antibodies successfully immunostained neurones from samples fixed with paraformaldehyde (Figures 1 and 2). It is widely known that the cross linkages in paraformaldehyde-fixed tissues are much weaker than those in tissues fixed with neutral buffered formalin, leading to a kind of fixation with the former that more effectively preserves tissues and cell surface antigens [22]. Thus, the antigens of intracellular Aβ would be preserved well and might be recognized by antibodies without formic acid treatment. It is also known that periodic acid pretreatment can improve immunoreactivity. These two factors may explain why we were able to detect intracellular Aβ even in brains from young monkeys.

In the brains of young monkeys, Aβ immunoreactivity was granular in nature. This diffuse pattern of immunoreactivity indicates that the intracellular Aβ in young monkeys may represent Aβ generated biologically in neurones, rather than accumulated Aβ that is typically found in neurones of aged animals (Figure 1**a**,**b**).

In the brains of aged monkeys, Aβ immunoreactivity in some cortical neurones tended to cluster together in clumps (Figure 1**c**,**d**). This clustering of immunoreactivity may represent Aβ accumulated in neurones and is consistent with previous hypotheses that Aβ accumulates in lipid rafts and that clustering of GM1 ganglioside within lipid raft-like membranes may facilitate the aggregation of Aβ (fibrils) in these rafts [23]. Although the pattern of Aβ immunostaining (i.e. intracellular Aβ) changed with age, we failed to find clear evidence that intracellular Aβ colocalizes with SPs.

In our previous studies, we described age-related changes of AD-related proteins in the cynomolgus monkey brain [19,20]. In the present study, we determined with biochemical methods whether intracellular Aβ accumulates with advancing age. In the microsomal fraction, the amount of total Aβ increased with age, whereas the amount of Aβ40 did not change with age (Figures 3 and 4**a**). Both Aβ42 and Aβ43, however, showed very interesting age-related changes. The amount of Aβ42 in the microsomal fraction increased significantly with age (Figures 3 and 4**a**). On the contrary, the amount of Aβ43 significantly decreased in this fraction (Figures 3 and 4**a**). This is the first study to show that the amounts of different Aβ molecules generated in the brain change with age of the individual. These results suggest that during ageing the cleavage of APP by γ-secretase may shift to generate more Aβ42 than Aβ43, which in turn may lead to increased accumulation of Aβ42 in the brain. Several studies have shown that Aβ42 is more closely associated with AD pathogenesis than is Aβ40 [24–27]. The age-related increase of Aβ42 observed in the present study supports these previous findings and is consistent with the hypothesis that increased Aβ42 generation or accumulation may promote, or even cause, sporadic AD pathogenesis in the aged brain.

As the nerve ending fraction prepared in the standard way contains both synaptic vesicles and synaptic plasma membranes, the amount of Aβ detected in this fraction may be extracellular Aβ associated with the outer part of the synaptic plasma membrane. To avoid potential contamination with extracellular Aβ, we pretreated each nerve ending fraction with Pro-K (this resulting fraction was termed the Pro-NE fraction). In the Pro-NE fraction, the amount of total intracellular Aβ significantly increased with age (Figures 3 and 4**b**). Our previous study showed that both full-length APP and βCTF accumulated in the nerve ending fraction with age [20]. It is noteworthy that AD-related proteins also significantly accumulate in the nerve ending fraction [19,20]. Thus, one possible explanation for this age-related accumulation of Aβ in the nerve ending fraction is that increased APP and βCTF in this fraction would subsequently cause intracellular Aβ to also accumulate in this fraction. Alternatively, the

age-related reduction of axonal transport to the soma for degradation may also cause the accumulation of these proteins, including Aβ, in the nerve ending fraction. Although the amounts of intracellular Aβ40, Aβ42 and Aβ43 significantly increased with age, the magnitude of increase varied for all three isoforms (Figures 3 and 4**b**). Of these three Aβ molecules, intracellular Aβ43 increased much less than did Aβ40 and Aβ42 (Figure 4**b**). This may explain why, in general, the generation of Aβ43 decreased with age (Figure 4**a**).

In summary, we found that intracellular Aβ exists in cortical neurones of monkeys as young as 4 years old, albeit in the form of diffuse granules. With advancing age (24–36 years old), however, intracellular Aβ tended to localize in neurones in the form of clusters or clumps (Figures 1 and 2). Our biochemical assays revealed that Aβ40 and Aβ43 were mainly generated in the brains of young monkeys. During ageing, the generation of intracellular Aβ shifted such that Aβ42 generation increased, while Aβ40 generation remained unchanged (Figures 3 and 4).

It is also noteworthy that the amount of intracellular Aβ increased in the nerve ending fraction with age (Figures 3 and 4), and this result closely parallels our findings with other AD-related proteins reported in our previous studies [19,20]. The accumulation of APP and βCTF in synaptosomes and/or synaptic plasma membranes may lead to Aβ overproduction in these compartments, and the accumulated intracellular Aβ, in turn, may be secreted extracellularly and accelerate SP formation. Thus, the nerve ending may be where SPs are initially formed or may be significantly associated with spontaneous SP formation that occurs with advancing age. Taken together, these results support the line of research that focuses in on age-related changes in the nerve ending. Findings from this line of research should reveal more details about the association between AD pathogenesis and other age-related neurodegenerative disorders.

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