Short Communication

E22Q-Mutant A β Peptide (A β Dutch) Increases Vascular but Reduces Parenchymal A β Deposition

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Patients that have hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) generate both wild-type β -amyloid (A β wt) and E22Q-mutant β -amyloid (A β Dutch). Postmortem analysis of HCHWA-D brains reveals severe cerebral amyloid angiopathy with very little parenchymal amyloid deposition. To investigate amyloidosis in the presence of both A β wt and A β Dutch variants, transgenic (tg) APP23 mice were crossed with APPDutch mice. Although single-tg APP23 mice deposited A β wt with aging, double-tg APP23/APPDutch mice co-deposited A β Dutch (mainly A β Dutch1-40) and A β wt at twofold higher total A β levels. Vascular A β deposits and hemorrhages were twice as high in APP23/APPDutch mice compared with APP23 mice. Surprisingly, parenchymal A β deposition was reduced in the double-tg mice compared with the single-tg APP23 mice. Our findings suggest that A β Dutch1-40 inhibits parenchymal amyloidosis but exacerbates vascular amyloid, hence explaining the compartment-specific distribution of cerebral amyloid in HCHWA-D patients. (*Am J Pathol* 2009, 174:722–726; DOI: 10.2353/ajpath.2009.080790)

Patients carrying familial mutations within the β -amyloid (A β) sequence of the amyloid precursor protein (APP) develop cerebral amyloid angiopathy (CAA) with or without additional parenchymal amyloid plaques.^{1,2} Because such patients are heterozygous for the mutation, they produce both wild-type (wt) and mutant A β . Several *in vitro* studies have demonstrated that mutated A β mixed with A β wt shows altered aggregation and toxicity compared with A β wt or mutated A β alone.¹ Nevertheless current transgenic (tg) mice that model familial cerebral amyloidosis caused by an APP mutation within the A β

sequence express only the mutant human A β but lack the complementary human A β wt counterpart.

Patients affected by hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) develop severe CAA and exhibit fatal hemorrhagic strokes typically in their 50s.³ These patients carry an E693Q APP mutation corresponding to E22Q of A β . To study an *in vivo* model that mimics HCHWA-D, we have crossbred APPDutch tg mice that express E22Q A β (A β Dutch)⁴ with homozygous APP23 mice expressing A β wt.⁵ Double-tg APP23/APPDutch mice were aged to 24 months and compared with single-tg APP23 littermates.

Materials and Methods

Transgenic Mice

The generation of APPDutch and APP23 tg mice has been described previously.^{4,5} APPDutch mice overexpress E693Q-mutated human APP751 whereas APP23 mice overexpress KM670/671NL-mutated APP751 (Swedish mutation). Both lines express their transgenes under the murine Thy-1 promoter element and have either been generated on a C57BL/6J background (APPDutch) or backcrossed to C57BL/6J mice for more than 10 generations (APP23). Homozygous APP23 mice were crossbred with hemizygous APPDutch mice. To distinguish double-tg APP23/APPDutch mice from single-tg APP23 mice levels of human APP and A β were assessed by Western blotting. A total of 23 mice was used: four female APP23/APPDutch versus eight female APP23 mice; and four male APP23/APPDutch versus seven male APP23. All animals were 24 months of age. The experiments were in compliance with protocols approved by the local animal care and use committees.

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Human Tissue

HCHWA-D tissue was from a 76-year-old male patient (provided by M.L.C. Maat-Schieman, Leiden University Medical Center, Leiden, The Netherlands). Tissue from a 61-year-old female with Alzheimer's disease (AD) was generously provided by M. Tolnay University of Basel (Basel, Switzerland).

Immunohistochemistry

Mouse brains were carefully removed while it was made sure that the leptomeningeal vessel remained attached to the brain. One hemisphere was immersion-fixed in 4% paraformaldehyde (the other half of the brain was used for Western blotting). Immunohistochemistry for A β was done on 25- μ m-thick, free-floating sagittal sections with polyclonal antibody NT12 (similar to NT11),⁵ using standard immunoperoxidase procedures (Vector Laboratories, Burlingame, CA). NT12 antibody has been shown to recognize both wild-type and Dutch-mutated A β .⁴

Quantification of Total A β Load and CAA

The A β load was quantified on A β -immunostained sets of every 12th systematically sampled serial sagittal 25- μ m-thick section (typically yielding 12 sections/hemibrain). Images of sections were acquired with a scanner (Perfection 3200 Photo; Epson, Long Beach, CA) using transmitted light and processed with Adobe Photoshop CS (Adobe Systems, San Jose, CA) and the area of the forebrain (reported in percent) covered by A β -immunostaining was determined with the Analyze Particles tool of ImageJ (National Institutes of Health, Bethesda, MA; <http://rsb.info.nih.gov/ij/>). Thus, A β load was defined as total parenchymal and vascular A β -immunostained profiles. Quantitative analysis of CAA was performed on the same NT12-immunostained sections. The number of A β -positive vessels throughout the neocortex and the overlying leptomeningeal vessels was counted manually using an Axioskop 2 microscope with a $\times 10$ objective (Zeiss, Oberkochen, Germany). A grading system dividing A β -positive vessels in one of three severity grades was applied as previously described.⁶ CAA grade 1: A β -immunoreactivity is restricted to the vessel wall; CAA grade 2: A β -immunoreactivity extends focally to the surrounding neuropil; CAA grade 3: extensive infiltration of A β into the parenchyma with a complete amyloid coat around the vessel. Neighboring profiles of immunoreactive vessel were considered as separate vessels.

Quantification of Cerebral Hemorrhage

Perls' Berlin Blue-stained clusters of hemosiderin-positive microglia were quantified on an additional set of every 12th systematically sampled sections⁶ throughout the neocortex and the overlying leptomeningeal vessels.

Western Blot Analysis

For analysis of total human A β and APP, we performed multiphasic urea/sodium dodecyl sulfate-polyacrylamide

gel electrophoresis and Western blotting. This gel system allows to separate A β 1-40 from A β 1-42 and A β wt from A β Dutch as described previously.^{4,7,9} Briefly, homogenized mouse brain hemispheres (forebrain) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% T, 5% C bicine/Tris minigels containing 8 mol/L urea in the separation gel. Proteins were transferred to a polyvinylidene difluoride Immobilon-P membrane by semidry blotting, incubated with antibody 6E10 (Covance, Princeton, NJ) and visualized by chemiluminescence (ECL; Amersham, Arlington Heights, IL). Antibody 6E10 recognizes residues 1 to 16 of A β , and the Dutch mutation at position 22 does not interfere with its binding. Synthetic A β Dutch was a gift of J. Ghiso (New York University School of Medicine, New York, NY). Intensities of both human A β and APP bands (4 μ l of 1:44 diluted sam-

Figure 1. APP and A β levels in brains of APP23/APPDutch and APP23 mice. **A:** Western blot analysis of human APP and A β in 24-month-old mouse forebrains ($n = 23$; females: four APP23/APPDutch and eight APP23 mice; males: four APP23/APPDutch and seven APP23). **Top:** APP levels in double-tg APP23/APPDutch mice (lanes 4 and 6) were at least twice as high compared with those in littermate single-tg APP23 mice (lanes 5 and 7). **Bottom:** Synthetic human A β wt1-40 and A β wt1-42 (lane 1), A β Dutch1-40 (lane 2), and A β Dutch1-42 (lane 3) peptides were used as markers. Whereas cerebral amyloid of single-tg APP23 mice was mainly composed of A β wt40 and with lower levels of A β wt42 (lanes 5 and 7), double-tg APP23/APPDutch mice showed an additional A β Dutch40 band (lanes 4 and 6) with an intensity similar to that of A β wt40. A β Dutch42 remained below detection level (lanes 4 and 6). The A β composition of an HCHWA-D brain (lane 8) was similar to that of APP23/APPDutch brains (lanes 4 and 6) with the exception that A β wt42 was readily detectable in the double-tg mouse brains. Western blot analysis of A β in AD brain (lane 9) resembled that of single-tg APP23 brains (lanes 5 and 7). **B and C:** Densitometric analysis of A β levels in the female mice revealed significant approximately twofold higher total A β 40 levels (composed of A β wt and A β Dutch) in double-tg APP23/APPDutch versus single-tg APP23 littermates (unpaired Student's *t*-test; *** $P < 0.001$) whereas A β 42 levels were similar in the two genotypes ($P > 0.05$). In male mice (results not shown) the increase in total A β 40 levels in APP23/APPDutch was 1.6-fold ($P < 0.01$) with again no change in A β 42 levels compared with APP23 littermates.

ples were loaded) were quantified with the Gel Analyzer Tool of ImageJ (<http://rsb.info.nih.gov/ij/>).

Statistical Analysis

Unpaired *t*-test was used for group comparisons using Microsoft Excel software (Microsoft Corporation, Redmond, WA). Values are reported as means $\pm$ SEM. Statistical significance is indicated as follows: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Results

Western blotting revealed that human APP levels in aged double-tg APP23/APPDutch mouse brain were at least twice those observed in single-tg APP23 mice (Figure 1A). While cerebral amyloid in APP23 mice was composed of both A β wt40 and A β wt42 with A β wt40 exceeding A β wt42 (Figure 1A), APP23/APPDutch mice exhibited an additional A β 40Dutch band in its intensity similar to A β wt40. The levels of A β Dutch42 remained below the limit of detection, similar to those previously reported for single-tg APPDutch mice.⁴ Consistently, quantitative analysis of total A β 40 (ie, wild-type and mutated A β combined) revealed an approximately twofold significant increase in APP23/APPDutch mice compared with APP23 mice, with no difference in total A β 42 (Figure 1, B and C).

A β immunohistochemistry of both APP23/APPDutch and APP23 brains revealed parenchymal and vascular A β deposition. Surprisingly however, total cerebral A β load in APP23/APPDutch mice was lower than that in APP23 littermates (Figure 2, A–C) despite the overall higher levels of total A β (Figure 1). In contrast, quantification of the number of A β -positive neocortical vessels (including the

overlying leptomeningeal vessels), revealed a nearly two-fold increase in APP23/APPDutch mice compared with APP23 mice (Figure 2D). We have previously shown that APPDutch mice at a similar age (22 to 24 months) develop only low levels of A β deposition, which is restricted to vessels.^{4,8} Previous quantification revealed only approximately nine A β -positive neocortical vessels (including the overlying leptomeningeal vessels) per section,⁸ which is only a quarter of the A β -immunopositive vessels in APP23 mice described in the present study. Vascular amyloid is a known cause of intracerebral hemorrhage in humans and APP tg mice^{2,6} and thus neocortical and leptomeningeal hemorrhages were additionally analyzed. Consistent with the increase in CAA, results revealed an approximately twofold increase in microbleedings in APP23/APPDutch mice compared with APP23 mice (Figure 2E).

Discussion

Cerebral amyloid in HCHWA-D patients consists of both A β Dutch and A β wt, with the vast majority being of the A β 40 species (present results and 4, 9, 10). In contrast, patients with AD reveal both A β 40 and A β 42 with A β 42 being the disease-driving and more toxic species.¹¹ Several studies have indicated that the ratio of A β 42 to A β 40 determines in which compartment cerebral amyloid deposition occurs, i.e., a high A β 42/A β 40 ratio promotes parenchymal amyloid deposition whereas a low ratio promotes CAA, and this appears to hold true for both A β wt and A β Dutch.¹ The reason why the A β 42/A β 40 ratio determines the cerebral compartment in which amyloid deposition occurs is not entirely clear. At least in transgenic mice using the Thy-1 promoter, cerebral A β is

Figure 2. Cerebral amyloidosis and hemorrhage in APP23/APPDutch and APP23 mice. The same mice as indicated in Figure 1 were used. **A–C:** Immunohistochemistry for A β revealed a significantly higher total A β load in the forebrains of female single-tg APP23 mice compared with female double-tg APP23/APPDutch mice (*n* = 8 and 4, respectively; unpaired Student's *t*-test; ***P* < 0.01) despite the observation that total A β levels were higher in double-tg compared with the single-tg mice (see Figure 1). The same was true for the male mice (*n* = 7 and 4, respectively; *P* < 0.05; not shown). **D:** Vascular A β load, however, was significantly higher in the female double-tg APP23/APPDutch mice compared with the single-tg APP23 mice, and this was true for total neocortical and leptomeningeal vessels (***P* < 0.01) as well as for individual cortical grade assessment C1 to C3 (*P* < 0.05). A similar increase in vascular A β load was found for the males (not shown). **E:** Quantification of neocortical and leptomeningeal hemorrhages revealed a significant, approximately twofold, increase in hemorrhages in female APP23/APPDutch mice versus APP23 mice (**P* < 0.05). Again, a similar increase was also found for the males (not shown). C, cortical; C1 to C3, cortical grade 1 to 3; L, leptomeningeal. Scale bar = 500 μ m.

neuron-derived and must subsequently be transported to the vasculature (possibly as part of its drainage along the periaxonal fluid pathway or into the blood for clearance).^{12,13} A β 42 aggregates more readily than A β 40 and thus, it is more likely to form an amyloid seed during its transport from the neuron to the vasculature.

The present finding that double-tg APP23/APPDutch mice develop fewer parenchymal A β deposits and significantly more CAA compared with single-tg APP23 mice reveals that the observed amyloid pathology in APP23/APPDutch mice is not a simple addition of the amyloid patterns seen in the corresponding single-tg mice. In particular, the reduced parenchymal A β deposition in APP23/APPDutch mice was unexpected in light of the higher levels of total A β in double-tg compared with single-tg mice. In addition, the twofold increase in vascular A β -deposits observed in APP23/APPDutch versus APP23 brains cannot be explained by a simple additive effect because 24-month-old single-tg APPDutch mice reveal only few A β deposits that are restricted to the vessels. Quantitatively, CAA in 24-month-old APPDutch mice is only a quarter of the amount found in single-tg APP23 mice.^{4,8} Although the (lower) A β 42/A β 40 ratio in the APPDutch/APP23 mice compared with the APP23 mice may be used to explain the increased CAA as well as the reduced parenchymal A β deposition, this explanation falls short if compared with single APPDutch mice, because APPDutch mice generate approximately eightfold more A β 40 compared with A β 42.⁴ Thus, on one hand the total A β generation seems to be an important variable. On the other hand, in familial AD, the A β 42/A β 40 ratio appears more important than the total A β to drive the deposition because some of the familial AD mutations in the presenilins specifically decrease A β 40 rather than increasing A β 42.¹⁴ From this and other observations,¹⁵ A β 40 was suggested to be inhibitory for parenchymal amyloid deposition. Indeed, *in vitro* A β 40 retards the aggregation of A β 42 in a concentration-dependent manner.^{16,17}

Introducing the E22Q substitution to A β results in a less efficient clearance of the mutated peptide from the brain, both by enzymatic cleavage and receptor-mediated transport into CSF/blood.¹ A β Dutch also shows a higher affinity to smooth muscle cells, where the initiation of vascular amyloid formation was suggested to occur.¹⁸ *In vitro*, A β Dutch reveals higher oligomerization and aggregation propensity compared with A β wt.^{19–21} Most relevant for the present study and the pathomechanism of HCHWA-D is the observation that mixing A β wt with A β Dutch alters and enhances aggregation propensities of either wild-type or mutated A β alone.^{1,22} Thus, it is likely that heterogeneous aggregates, protofibrils, or fibrils made of both A β Dutch and A β wt have different biological propensities compared with homogeneous A β species made of either wild-type or mutated A β alone. The mouse models used in the present study also express a small amount of murine A β and thus, it cannot be ruled out that murine A β additionally influences the biological properties of the human A β species.

In conclusion, our results suggest that the expression of E22Q-mutated A β (primarily A β Dutch40) is inhibitory

for parenchymal amyloidosis but exacerbates vascular amyloidosis, therewith explaining the compartment-specific distribution of cerebral amyloid observed in HCHWA-D patients. The finding that the amyloid lesions in APP23/APPDutch mice are not a simple overlay of the amyloid lesions in the corresponding single-tg mice suggests that A β wt and A β Dutch interact in mice as well as in HCHWA-D patients.

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