ApoE deficiency leads to a progressive age-dependent blood-brain barrier leakage

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Increasing evidence points toward a vascular component in the pathophysiology of age-related neurodegenerative diseases, such as AD (22, 25, 47). For instance, immunological studies in AD patients show the presence of plasma proteins in the brain parenchyma (25). It is conceivable that defects in the barrier function over time would contribute to this accumulation of blood proteins in the brain parenchyma. Although subtle morphological BBB changes occur with age (25), significant functional, age-related BBB changes in the absence of disease have thus far not been described (3, 32, 39). Therefore, it is of interest to systematically investigate the effect of aging on the integrity of this important organ. Here, we investigate the extent of BBB leakage in WT and apoE−/− mice as a function of age. Furthermore, to determine the contribution of tissue- vs. blood-borne apoE to vascular permeability, we generated chimeric mice by bone marrow transplantation and measure their BBB leakage.

MATERIALS AND METHODS

Mice. WT and apoE−/− mice (30) on C57BL/6J background (Jackson Labs) were bred to various ages. Mice were fed normal chow (Prolab 3000, PMI Nutrition). Plasma cholesterol levels were measured in apoE−/− mice using the Cholesterol-20 kit to confirm the genotype (6). Experiments adhered to national and institutional guidelines in the care and use of animals. The animal protocols were approved by the Massachusetts Eye and Ear Infirmary’s Institutional Animal Care and Use Committee.

Vascular leakage. Vascular leakage was quantified using the established Evans blue (EB) dye technique (18, 44), which can be as sensitive and quantitative as the isotope-dilution method when measuring albumin leakage (43), though the isotope-dilution technique can distinguish the leakage of a wider range of molecules. EB dye was injected intraperitoneally (50 μg/g body wt). Distribution of the dye was confirmed by a visible change in the mouse skin color within 1 h after injection. Three hours after receiving injections, the mice were anesthetized with a 1:1 ketamine and xylazine mixture (100 and 10 mg/kg body wt, respectively) and blood was obtained for measurements of the plasma EB levels. Immediately afterward, the mice were euthanized by decapitation. Absorptive tissues were then used to soak up all exuding blood from the head. The skull and the highly vascularized meningeal layer were microsurgically opened, all traces of blood were absorbed with tissues, and the brain hemispheres and cerebellum were collected in their entirety and placed separately in preweighed and labeled tubes. The tubes were then reweighed to obtain the tissue weights, as previously described (19, 24). Five hundred microliters of formamide (Sigma, St. Louis, MO) were added to each of the tissue containing tubes to extract the EB. The tubes were then centrifuged, and the absorbance at 620 nm was measured.

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covered and stored in the dark for 72 h. The supernatant was then obtained and centrifuged to remove particles from the suspension. Subsequently, the optical density (OD) of the extracted dye was measured at 620 nm by spectrophotometry. In some experiments, as indicated, intravascular content was removed by cardiac perfusion before the tissue harvest, as previously described (43). Briefly, animals were anesthetized, the chest cavity was surgically opened, and a 14-gauge perfusion cannula was introduced into the aorta. Drainage was provided from the right atrium. Mice were perfused with 10 ml of PBS to remove intravascular content.

Plasma readings. To ensure that equivalent concentrations of EB dye were absorbed from the peritoneum into the circulation of all groups of mice studied, a blood sample from each mouse was obtained just before tissue harvest, and the OD of the dye in the plasma was measured by spectrophotometry. The average OD of the dye in the plasmas from each group of mice in all our experiments was not significantly different, as indicated in the following representative example from the aging experiments depicted in Fig. 3: [ODaverage(Wt) = 0.19 ± 0.009, n = 48; ODaverage(ApoE−/−) = 0.188 ± 0.006, n = 43; P = 0.4], ensuring that the differences in BBB permeability were not due to differences in plasma EB concentration.

Calibration curves. To determine the concentration of the dye absorbed into the circulation of mice in our experiments, first a calibration curve was generated, showing the OD at 620 nm of increasing concentrations of EB dye in a pool of plasma obtained from three WT mice. Similarly, to calculate the EB concentration from the ODs of the formamide tissue extracts, we generated a calibration curve with increasing concentrations of EB dye in formamide. The standard curves showed a linear relationship between the concentration of EB dye in WT plasma or formamide and the corresponding OD within the measured range (Fig. 1A). Using the curves, we determined that the concentration corresponding to the average optical density reading of the EB dye in the plasmas of any group of mice from all of our experiments corresponds to a 2 μg/ml concentration after ×100 dilution from plasma. Therefore, the average concentration of EB dye absorbed into the mouse circulation 3 h after an initial intraperitoneal injection of 50 μg/g body wt EB dye is ~200 μg/ml.

Determining maximum EB protein-loading capacity. To confirm that the concentration of injected EB dye was predominantly bound to plasma proteins, we passed 50 μl of mouse plasma containing various concentrations of EB dye through 30K nanofilters (Millipore) and measured the OD of the dye in the filtered fluid. Since the filter hinders passage of most plasma proteins, the amount of EB dye detected in the filtered fluid reflects the EB not bound to plasma proteins. This reveals the plasma concentration at which the protein binding of EB is saturated. Up to a concentration of 500 μg/ml no EB was detectable in the filtered fluid (Fig. 1B), which indicates that the concentration used in our mouse experiments (average plasma EB concentration of 200 μg/ml) would result in a total binding of EB dye to the plasma proteins.

Bone marrow transplantation. For the preparation of bone marrow, donor mice were euthanized; femur and tibia bones were isolated under sterile conditions and the bone marrow cells were harvested by flushing the bones with medium (RPMI, 5% fetal bovine serum). The cells were washed and resuspended in Hanks’ balanced salt solution. Recipient 6-wk-old male mice were lethally irradiated by exposure to a cesium source, two doses of 6 Gy, 3 h apart. Immediately after the second dose of irradiation, the mice were injected with 1 × 107 freshly prepared donor bone marrow cells. For the first 4 wk after transplantation, recipient mice were kept in a sterile unit and given sterile water and sterile food. Eighteen weeks after transplantation, the mice were used in the experiments. To determine the reconstitution of the bone marrow cells in the irradiated hosts, a complete blood cell count of heparinized blood was performed using an automatic cell counter (Coulter, Miami, FL). To confirm the successful repopulation of bone marrow cells in the transplanted animals, we performed intracellular staining of apoE, since peripheral blood monocytes contain apoE (45). First, the leukocytes were immobilized on slides. The cells were then fixed with 4% Formalin. Next, the membranes were permeabilized with 1% Triton and finally were stained with a primary goat anti-apoE polyclonal antibody (Research Diagnostics; catalog no. RDI-APOEαgX) and a secondary labeled antibody (rabbit anti-goat IgG FITC polyclonal antibody; ICN, catalog no. 55348).

Statistics. Comparisons of groups were performed using paired or unpaired Student’s t-test where appropriate. Analyses of the age-dependent changes in leakage were performed by regression analysis and trend lines were calculated, with equations indicated in the legends. All analyses were performed using Microsoft Excel software. Values of P < 0.05 were considered statistically significant.

Fig. 1. Validation and optimization of the experimental Evans blue (EB) technique for the study of blood-brain barrier (BBB) permeability. A: calibration curves were generated to obtain concentrations of the EB dye absorbed into the plasmas and tissues of mice from our experiments. To do so, the optical density (OD) of increasing EB concentrations in mouse plasma (bottom line, ●) or formamide (top line, ○) was measured by spectrophotometry. B: to determine the plasma saturation concentration of EB dye, mouse plasma containing a range of EB concentrations was passed through 30 K nanofilters to separate unbound from protein bound EB. The protein bound EB does not pass through the nanofilter, therefore, the OD of the postfiltration fluid reflects unbound EB. Single arrow, average EB concentration in the plasmas of the mice in our aging experiments 3 h after intraperitoneal injection. Double arrow, the plasma-EB concentration at which EB becomes detectable in the filtered fluid. These measurements confirm that our plasma EB concentrations are below the saturation of the plasma proteins and therefore leakage of unbound EB through the BBB is unlikely.
RESULTS

To investigate the impact of aging on vascular integrity, we measured the amount of EB dye leakage in the brain tissues of WT mice of mixed genders and various ages (Fig. 2). To account for possible differences between the various brain regions, we examined each hemisphere and the cerebellum separately. Surprisingly, there was a subtle yet significant increase in EB leakage with increasing age in each cortical hemisphere using analysis of regression (\( n = 75, r^2_{\text{left hemisphere}} = 0.33, P < 0.01; n = 75, r^2_{\text{right hemisphere}} = 0.23, P < 0.01 \)), suggesting an impairment of the BBB in WT mice as they age, with a notable amount of the permeability increase occurring during the time of sexual maturity, i.e., around the age of 100 days (Fig. 2).

In addition to our analysis of regression, we directly compared the cerebral leakage of different age groups (\( \text{age}_{\text{average}} = 32 \) days, \( n = 8; \text{age}_{\text{average}} = 103 \) days, \( n = 13; \text{age}_{\text{average}} = 378 \) days, \( n = 12 \)). Our results showed that the average leakage in the cerebral cortex of each age group was significantly different from that of the other age groups, with an increase of leakage with age (\( \text{OD/gram}_{2\text{days}} = 0.43 \pm 0.02; \text{OD/gram}_{103\text{days}} = 0.61 \pm 0.04; \text{OD/gram}_{378\text{days}} = 0.81 \pm 0.05; \text{P}_{32\text{vs}103\text{days}} < 0.01; \text{P}_{103\text{vs}378\text{days}} < 0.01; \text{P}_{32\text{vs}378\text{days}} < 0.01 \)). A similar pattern of results was found in the cerebellum (\( \text{OD/gram}_{2\text{days}} = 0.64 \pm 0.1; \text{OD/gram}_{103\text{days}} = 0.82 \pm 0.06; \text{OD/gram}_{378\text{days}} = 1.08 \pm 0.06; \text{P}_{32\text{vs}103\text{days}} = 0.11, \text{P}_{32\text{vs}378\text{days}} < 0.01; \text{P}_{103\text{vs}378\text{days}} < 0.01; n, \text{numbers same as for cerebral cortex} \).)

Although previous reports show that intravascular blood volume does not significantly change with age (1, 20, 23, 40, 41) and that differences in intravascular content may account for the vascular changes we observed in the above experiments by performing an established cardiac perfusion technique (43) before tissue harvest in EB-injected WT mice of two different age groups. In perfused WT mice the average EB leakage of both cerebral and cerebellar cortex significantly increased with age (\( \text{age}_{\text{average}} = 77 \) days, \( n = 7; \text{age}_{\text{average}} = 161 \) days, \( n = 6; \text{P}_{77\text{vs}161\text{days}} < 0.01 \)), confirming the above-mentioned subtle changes to the BBB (Fig. 2D).

Adult apoE\(^{-/-}\) mice have a defect in their BBB (9, 24, 28). However, until now, the degree of impairment as a function of age has not been systematically studied. Using apoE\(^{-/-}\) mice of mixed genders and various ages, we found a strikingly high correlation between leakage and age in the cortical hemispheres (\( r^2_{\text{left hemisphere}} = 0.74, P < 0.01; r^2_{\text{right hemisphere}} = 0.63; P < 0.01 \)) and the cerebellum (\( r^2_{\text{cerebellum}} = 0.65; P < 0.01 \)) of the apoE\(^{-/-}\) mice (\( n = 43 \)) (Fig. 3).

In addition to our analysis of regression, we directly compared the average cerebral leakage of different age groups in the apoE\(^{-/-}\) mice. Our results showed that the average leakage in the cerebral cortex of the older age group was significantly higher than that of the younger age group (\( \text{age}_{\text{average}} = 31 \) days, \( n = 13; \text{OD/gram}_{31\text{days}} = 0.49 \pm 0.03; \text{age}_{\text{average}} = 103 \) days, \( n = 11; \text{OD/gram}_{103\text{days}} = 1.06 \pm 0.05; \text{P}_{31\text{vs}103\text{days}} < 0.01 \)). A similar result was found in the cerebellum when comparing a younger and older group of apoE\(^{-/-}\) mice.
mice (ages and n numbers same as for cerebral cortex, OD/ Gram31days = 0.58 ± 0.04, OD/Gram103days = 1.46 ± 0.11, P31 vs 103 days < 0.01).

Furthermore, we find that the younger mice are not significantly different in their EB leakage compared with their WT counterparts (n apoE−/− = 9, ageaverage = 31 days ± 0, OD/Gramleft hemisphere = 0.45 ± 0.02, OD/Gramright hemisphere = 0.4 ± 0.02, OD/Gramcerebellum = 0.56 ± 0.06; nWT = 9, ageaverage = 32 days ± 1.2, OD/Gramleft hemisphere = 0.43 ± 0.02, OD/Gramright hemisphere = 0.41 ± 0.03, OD/Gramcerebellum = 0.6 ± 0.1; Pleft hemisphere = 0.4, Pright hemisphere = 0.76, Pcerebellum = 0.74). However, aged apoE−/− mice showed significantly higher leakage compared with their age-matched WT counterparts. (n apoE−/− = 9, ageaverage = 108 ± 6 days, OD/Gramleft hemisphere = 1.1 ± 0.05, OD/Gramright hemisphere = 1.0 ± 0.05, OD/Gramcerebellum = 1.5 ± 0.12; nWT = 18, ageaverage = 108 ± 4 days, OD/Gramleft hemisphere = 0.64 ± 0.03, OD/Gramright hemisphere = 0.66 ± 0.04, OD/Gramcerebellum = 0.84 ± 0.06; Pleft hemisphere < 0.01, Pright hemisphere < 0.01, Pcerebellum < 0.01), suggesting a more progressive compromise in BBB function in the apoE−/− mice. Indeed, with increasing age apoE−/− mice showed a significant 3.7× higher rate of leakage compared to WT.

Since previous studies have indicated lateralization of BBB function under certain conditions (29, 36), we measured the leakage in the hemispheres separately. The left cortical hemisphere in both WT and apoE−/− mice across age showed no significant difference in the amount of leakage to that in the right cortical hemisphere (Figs. 2 and 3). However, the amount of cerebellar leakage was surprisingly significantly higher in each WT and apoE−/− mouse compared with the leakage in each animal’s respective cerebral cortices across age (Figs. 2 and 3). Consistent with these data in nonperfused mice, the cerebellar leakage in the perfused WT mice was significantly higher than that of the cerebral cortices (P < 0.01) (Fig. 2D).

Gender and leakage of brain vessels. Since apoE-dependent age-related cognitive decline mostly affects women (27), we further examined our mice for potential gender differences in the amount of BBB permeability. Our results did not show a significant difference in the amount of EB leakage through the BBB between the male WT (ageaverage = 114 ± 13 days and n = 37 for each brain region) and female mice (ageaverage = 114 ± 14 days and n = 20 for each brain region - OD/Grammale left hemisphere = 0.64 ± 0.03, OD/Gramfemale left hemisphere = 0.6 ± 0.03, P = 0.45; OD/Grammale right hemisphere = 0.65 ± 0.03, OD/Gramfemale right hemisphere = 0.57 ± 0.03, P = 0.1; OD/Grammale cerebellum = 0.85 ± 0.06, OD/Gramfemale cerebellum = 0.86 ± 0.06, P = 0.95).

Leakage of brain vessels in bone marrow-transplanted mice. Since apoE is expressed in various cells, we wondered whether the lack of apoE in tissue or in leukocytes contributes to the increase in EB permeability. To investigate this question, we generated chimeric mice by bone marrow transplantation, WT for their blood cells and apoE−/− elsewhere and vice versa. To confirm the successful repopulation of bone marrow cells in the transplanted animals, we stained peripheral blood leukocytes for apoE (Fig. 4A). Only animals confirmed to be successfully transplanted were used for the experiments. WT → WT and apoE−/− → apoE−/− transplanted animals showed a similar pattern of experimen-
tal results as WT and apoE−/− mice, respectively (Figs. 2, 3, 4B), whereas the chimeric animals, apoE−/− → WT and WT → apoE−/−, both showed significantly less EB permeability than apoE−/− → apoE−/− and more than WT → WT (Fig. 4B).

DISCUSSION

Current opinion suggests that in healthy individuals the overall BBB function remains stable throughout life (33, 39). However, in this study, we show that in WT animals the BBB function significantly deteriorates with age. This finding proposes that the BBB, similar to other organs of the body, is subject to changes resulting from age. It is conceivable, however, that developmental milestones, such as sexual maturity, may contribute to the BBB changes with age. In line with our findings, various morphologic changes in the microvessels during aging are reported, including thinning of the capillary walls of the white matter due to pericyte loss, thinning of the endothelial cytoplasm (37), and age-related changes in tight junctions (26), which could underlie the differences in age-related leakage. Furthermore, consistent with Fullerton et al. (9), we find a significantly higher leakage in the cerebella of both WT and apoE−/− mice when compared with their cerebral cortices. Previous reports (7, 42) indicate ~12% lower blood volume in the cerebral cortex compared with that of the cerebellum, which may in part contribute to the higher BBB leakage in the cerebellum, since a higher vascular surface area in that organ would give intravascular molecules a higher chance to cross the barrier and invade the parenchyma. However, the small differences in the aforementioned studies do not explain the relatively large differences in leakage in our study (up to twofold higher than cerebral cortex), especially since the differences remain after tissue perfusion. Hence, our results suggest that vessels of the cerebellum have genuinely different BBB properties compared with the vessels of the neo-cortex and that the increased leakage is not solely due to the differences in blood volume in these organs. These findings may help in determining optimal drug dosages or understanding mechanisms of cerebellar diseases, such as ataxia.

Heretofore, age-related functional changes of the BBB may have remained undetected partly due to the lack of a systematic examination across age, the methods employed in the previous studies, the inherently high biological variability of the measured entity, or the low number of animals used in the previous examinations. For example, in previous studies (32) of the BBB and age, the marker or tracer was mostly administered for short time periods (i.e., 10 min to 1 h), which may not have been long enough to detect differences in leakage between groups, for example, such as those found in our study by waiting to harvest tissues 3 h after administration of the EB dye.

Elucidating the brain’s vascular changes with age is important, since a growing body of evidence points toward vascular pathology during age-related neurodegenerative diseases (12, 13, 25, 47), such as leakage due to a compromised BBB (15, 25). Up to now, these changes have mostly been interpreted as a probable result of the disease. In contrast, our results suggest that age-related changes in the BBB occur in healthy animals and therefore could precede and perhaps even be a cause of neurodegeneration. Indeed, our data show that a notable amount of the permeability increase occurs in mature but not yet old mice, i.e., around the age of 100 days. Since the BBB is a selective physical interface between the neurons and the intravascular content, it is conceivable that losses in the barrier function over time may result in neuronal damage, for instance through exudation and accumulation of plasma proteins, such as amyloid, in the interneuronal space. This hypothesis may offer new venues for prevention of neurodegenerative diseases.

Interestingly, two of the most important risk factors for prominent neurodegenerative diseases, such as Alzheimer’s disease (AD) and age-related macular degeneration (AMD), are age (5, 35, 47) and apoE4 (38). In line with these facts, apoE4 transgenic mice develop drusenoid retinal depositions and spontaneous choroidal neovascularization, common features of AMD (21). Although the details of how aging or apoE may lead to AD or other age-related neurodegenerative diseases are not well understood, our work suggests a plausible link through the importance of apoE for BBB maintenance.

However, how apoE may lead to BBB dysfunction is unknown. Some reports speculate that when apoE binds to a receptor it initiates intracellular signaling pathways that keep the BBB intact (9). It is also conceivable that the function of astrocytes, which are a crucial component of the BBB (16) and which are also known to generate apoE (4), may be disrupted by the lack of apoE. Such a disruption of astrocyte function might thereby impede the induction of the BBB properties.

Another plausible explanation of how lack of apoE contributes to the BBB dysfunction may be due to inflammatory processes. ApoE−/− mice are prone to chronic inflammatory conditions that progress with age (31), such as atherosclerosis
(46). We hypothesize that apoE−/− leukocytes have a higher activation status, which may cause them to be more prone to attack the vascular wall. Both acute and chronic inflammation cause disruption of the BBB and blood retinal barrier, i.e., after trauma (24) or during diabetic retinopathy, respectively (17). These facts lead us to speculate that constitutive inflammatory processes during physiological aging could cause cumulative damage that compromises BBB function, which would be in line with our finding of a subtle increase in BBB leakage in WT animals with age. Indeed, neutrophils release during CD18/CD11b and ICAM-1 mediated interaction with the endothelium a highly potent permeability factor (10), the inactive serine protease azurocidin, which can cause massive leakage in peripheral non-CNS vessels (11). However, it is unknown whether azurocidin can also mediate BBB/blood-retinal barrier leakage (14).

Another indication for immune cell involvement in BBB dysfunction is revealed by our bone marrow transplant experiments. The results of these experiments suggest that BBB homeostasis depends on equal contributions from tissue and blood cell derived apoE. The fact that the level of BBB permeability is significantly increased when the apoE derived from blood cells is absent reinforces the putative role for leukocytes in BBB maintenance. However, it remains to be investigated how apoE from blood cells may contribute to the integrity of the BBB, or why lack of apoE may lead leukocytes to cause BBB breakdown.

In summary, this work shows the gradual and continuous decline in BBB function with physiological aging, a process exacerbated in apoE−/− mice. We provide evidence that immune cell-derived apoE is important in maintaining BBB integrity. These results lead us to hypothesize that constitutive inflammatory processes during aging cause cumulative damage to the BBB, which may be a prelude to age-related neurodegenerative diseases.

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