Elderly apolipoprotein E$^{-/-}$ mice with advanced atherosclerotic lesions in the aorta do not develop Alzheimer's disease-like pathologies

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Abstract. Atherosclerosis and Alzheimer's disease (AD) are a major cause of morbidity and mortality in Western societies. These diseases share common risk factors, which are exhibited in old age, including hypertension, diabetes, hypercholesterolemia and apolipoprotein (Apo) ε4 allele. We previously demonstrated that factor XI (FXI) deficiency in mice reduced the atherosclerotic plaque area in coronary sinuses and the aortic arch. This led us to investigate whether FXI deficiency in elderly ApoE knockout (KO) mice would decrease pathological alterations compatible with atherosclerosis and AD. The present study used ApoE/factor XI double KO (ApoE/FXI DKO) mice aged 64 weeks and age-matched ApoE KO mice to serve as a control group. The ApoE KO mice developed an advanced atherosclerotic lesion area in the aortic arch, which was reduced by 33% in the DKO mice. However, neither atherosclerosis nor AD-associated pathological alterations in the elderly mice brains were observed in either the DKO mice or the ApoE KO mice. The results advocate a dichotomy between the brain and peripheral blood vessels. Therefore, the ApoE KO and DKO mice cannot serve as mouse models for studying AD or pathological brain changes compatible with atherosclerosis. The mechanism by which ApoE KO protects against brain pathology should be further studied as it may prove helpful for future treatment of senile dementia.

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Introduction

Atherosclerosis is a major cause of morbidity and mortality in Western societies (1,2). It is a chronic inflammatory disease of the arterial wall, which is characterized by lipid accumulation, leukocyte infiltration and smooth muscle cell proliferation. Atherosclerosis is considered to be a risk factor for cognitive deterioration in the elderly, including Alzheimer's disease (AD) (3,4). The latter is a neurodegenerative disease, which is characterized by dementia, along with a dense deposition of amyloid-β (Aβ) protein in senile plaques in the brain, hyperphosphorylated tau protein and neuron loss. Several findings suggest that atherosclerosis and AD are linked: i) Atherosclerotic vascular disease and AD share common risk factors, such as hypertension, diabetes, hypercholesterolemia, and apolipoprotein ε4 allele (5). In addition, old age is a major risk factor for both atherosclerosis and AD (6). ii) There are reports of a correlation between carotid atherosclerosis and AD (7) as well as atherosclerosis of the circle of Willis and AD (8,9). Moreover, coronary artery disease is increased in AD patients (10). iii) In a transgenic mouse model of AD (B6Tg2576), early atherosclerosis lesions were detected and were positively correlated with cerebral β amyloid deposits when mice were fed a normal diet (11) or atherogenic diets (12). iv) Brain cholesterol affects the Aβ formation from amyloid precursor protein (APP) (13,14). v) APP and Aβ are present in human carotid plaques (15). vi) APP transgenic mice with apolipoprotein E (ApoE) deficiency had increased atherosclerosis and vascular inflammation (16). However, whether atherosclerosis contributes to AD or they just share similar epidemiology is still not known. ApoE is the main lipid carrier protein in the brain, and it is released by astrocytes in order to supply neurons with cholesterol. The human ApoE gene has 3 allelic variants (ε2, ε3, and ε4). The ε4 allele has been associated with a higher risk of cardiovascular disease and AD, while the ε2 allele has a protective effect against AD. In mice, the association between ApoE deficiency and AD is not clear (17-26). Recent findings from our group and others demonstrate the involvement of coagulation factors in both

atherosclerosis (27,28) and AD (29,30). The contact activation pathway plays an essential role in hemostasis, and also in the progression of thrombosis and inflammation (31). In addition, factor XII, the initiator of contact activation, has been shown to be activated by aggregated Aβ (30), which resulted in activation of factor XI (FXI), thus enhancing brain thrombin generation. In humans, increased FXI is associated with increased incidence of ischemic stroke (32), whereas subjects with severe FXI deficiency have a reduced incidence of ischemic stroke (33). High levels of thrombin were found in the circulation, brain parenchyma and micro-vessel walls of AD patients and also in AD mouse models (30,34,35). In addition, high levels of fibrinogen are correlated with increased cerebral amyloid angiopathy and microglial activation when assessed in AD mouse models (36). Procoagulant proteins, including FXI, were found adjacent to macrophages and smooth muscle cells inside atherosclerotic plaques (27,37). Deprivation of FXI in ApoE knockout (KO) mice aged 24 and 42 weeks resulted in a significant reduction of atherosclerosis in the aortic sinus and aortic arch in comparison to ApoE KO mice (28). Targeting FXI prevented thrombosis on acutely ruptured atherosclerotic plaques (38). Furthermore, APP and Aβ were found in advanced human carotid plaques, in proximity to activated macrophages and platelets (15). Hence, it can be suggested that coagulation factors, including FXI, play a role in both atherosclerosis and AD. Old age is the main risk factor for AD; therefore, in this work, we sought to study whether FXI deficiency in elderly ApoE KO mice would decrease pathological changes compatible with atherosclerosis and AD.

Materials and methods

Mice. Male ApoE KO mice (n=12; Jackson Laboratory, Ben Harbor, ME, USA) and male ApoE/FXI double knock out (DKO) mice (n=10) (28) were included in the study. The mice fed with a chow diet and water provided ad libitum, throughout the experiments. Mice were sacrificed at 64 weeks of age and organs were collected for further analysis. All procedures were approved by the Institutional Animal Care and Use Committee of the Sheba Medical Center.

Atherosclerosis assessment. Quantification of atherosclerotic lesions was performed by calculating the lesion area in the aortic arch. The aorta was removed from the aortic arch to the iliac branches and fixed in 4% formalin. The aorta was cut longitudinally and stained with Sudan IV (Fluka). Lesion-area analysis in the thoracic aorta was conducted blind, using NIS Elements imaging software (Nikon Corporation, Tokyo, Japan).

Systemic inflammation. For plasma preparation, blood was drawn from the vena cava in tubes with 10% EDTA. Plasma was separated by centrifuge (1,000 x g) for 10 min, and stored at -80ºC until analysis was performed. The interleukin-6 (IL-6) concentration was measured by enzyme-linked immunosorbent assay (ELISA) kit (EMD Millipore, Billerica, MA, USA).

Brain tissue preparation. Brain specimens were harvested, hemi-dissected, and one hemisphere was fresh-frozen for histological and morphological studies. The frontal and parietal cortices, hippocampus and cerebellum were dissected from the other hemisphere. Specimens were frozen rapidly in liquid nitrogen and stored at -80˚C until analysis. Slices of brain from 5XFAD transgenic male mice were kindly provided by Dr Frenkel D. (Tel-Aviv University, Israel).

Cortex cytokines levels. Cortices were homogenized in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplement with protease inhibitors. The homogenate was centrifuged at 13,000 x g for 18 min. Quantitation of total protein in the extract was measured by Micro BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Tumor necrosis factor-α (TNF-α) and IL-6 concentrations were measured using ELISA kits (EMD Millipore).

Histology. Frozen brains were serially sectioned to slices of 10-μm thickness using a cryostat (Leica CM 1850; Leica Microsystems GmbH, Wetzlar, Germany). The histologic sections were stained with H&E. The Aβ deposits in the brain were detected using a Congo-red staining kit (Ventana Medical Systems, Inc., Tucson, AZ, USA). For microglia immunostaining, brain slices were fixed with 4% paraformaldehyde rinsed in PBS [0.1 M (pH 7.2)]. The slices were permeabilized and blocked with 0.1% Triton X-100/PBS (PBST) containing 10% normal serum to reduce the non-specific adherence of antibodies. Brain slices were incubated in primary antisera [goat anti-ionized Iba 1 (1:100; Abcam)] for 1 h at 25˚C in a humid chamber. After incubation with the primary antisera, the slices were rinsed with PBST and incubated with anti-goat Alexa Fluor-568 conjugated secondary antibody (Molecular Probes; Thermo Fisher Scientific, Inc.). Slices were rinsed with PBST 3 times. Sections were then rinsed 3 times with PBST and cover slipped with fluoromount mounting medium (Sigma-Aldrich Israel, Ltd., Rehovot, Israel). The sections were observed with x20 magnification under an aBX-43 fluorescence microscope (Olympus Corporation, Tokyo, Japan). Color pictures were acquired and analyzed using a digital camera system coupled to imaging software (cellSens Entry digital imaging software; Olympus Corporation) under a constant exposure time, gain and offset, which were chosen in order to increase the threshold for fluorescence.

Results

Atherosclerosis. To investigate the effect of FXI deficiency on atherosclerosis in elderly mice, we measured the atherosclerotic lesion area at the aortic arch of 64-week-old ApoE KO and FXI-deficient DKO mice. As expected, in the aortic arch, the elderly ApoE KO mice had a higher proportion of atherosclerotic lesion area (~30%, n=10) compared to the FXI-deficient DKO mice (~20%, n=5) (Fig. 1). As the elderly ApoE KO mice presented advanced aortic atherosclerosis, we assumed that they would develop pathological changes in the brain and that FXI deficiency would inhibit these pathological changes. Therefore, we analyzed whether the ApoE KO mice had suffered from brain atrophy and modification of hippocampus size in comparison to DKO mice. Unexpectedly, hemotoxylin

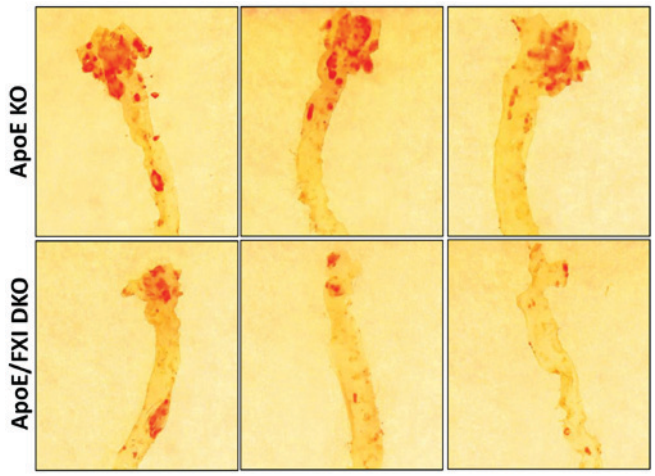

Figure 1. Atherosclerosis in aorta. Atherosclerotic lesion area of ApoE KO (n=10) compared with ApoE/FXI DKO mice (n=5) at 64 weeks of age. The atherosclerotic lesion was stained with Sudan IV. Apo, apolipoprotein; KO, knockout; ApoE/FXI DKO, ApoE/factor XI double KO; FXI, factor XI.

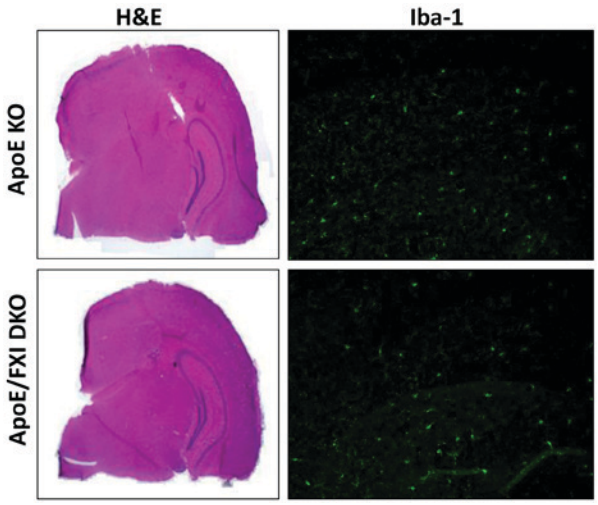

Figure 2. Brain pathologies. Brain morphology and microglia cells in the brains of ApoE KO (n=5) compared with ApoE/FXI DKO mice (n=5) at 64 weeks of age. Brain sections were stained with H&E (left panel) or immunostained with Iba-1 (right panel). Apo, apolipoprotein; KO, knockout; ApoE/FXI DKO, ApoE/factor XI double KO. FXI, factor XI.

and eosin (H&E) staining showed no pathologic damage to the brain in these very elderly ApoE KO mice (Fig. 2), and there was a histological similarity between the ApoE KO and DKO mice brains.

Inflammation. To investigate neuro-inflammation, cortex cytokines and microglia levels were measured. ApoE KO and DKO mice had similar levels of IL-6 (1.3 vs. 1.7 pg/mg protein) and TNF-α (2.21 vs. 2.07 pg/mg protein) in the brain cortex. Similar densities of microglia were detected in the brain sections from ApoE KO and DKO as demonstrated by ionized calcium binding adapter molecule 1 (Iba-1) staining (Fig. 2).

Changes compatible with AD. Congo red staining was used to measure the deposition of Aβ in the brain. Compared to 5XFAD transgenic male mice, which showed patchy deposits of Aβ in the brain tissue, elderly ApoE KO mice had no Aβ lesions in the brain (Fig. 3).

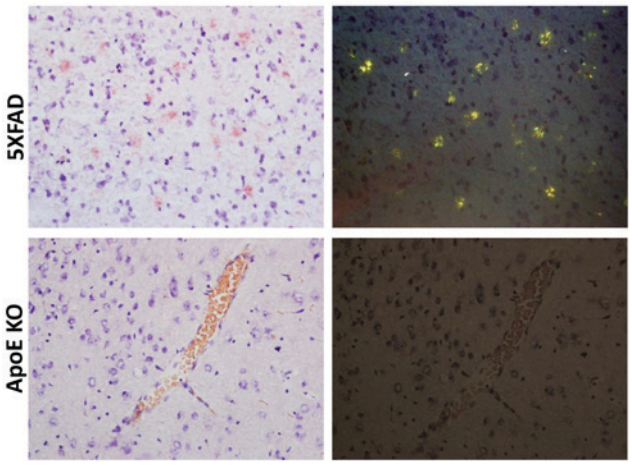

Figure 3. AD-like pathologies. Brain section from 5XFAD transgenic mice and ApoE KO mice stained with Congo red. The left panel shows brain tissue with patchy deposits of congophilic, orange-stained material, representing amyloid. The right panel shows a typical birefringent apple-green stain of the amyloid deposits examined with polarized light. (Congo Red stain, x400). AD, Alzheimer's disease; Apo, apolipoprotein; KO, knockout.

Discussion

Previous studies have suggested that the coagulation pathway is involved in both atherosclerosis and AD (28,30). This motivated us to investigate the influence of FXI deficiency on atherosclerosis and pathological brain changes associated with AD in very old ApoE KO mice. Our results show that: I. although elderly ApoE KO mice develop advanced aortic atherosclerotic lesions, they do not develop senile Aβ plaques in the brain, and II. FXI deficiency reduces the aortic atherosclerotic burden, and similar to ApoE KO mice, ApoE/factor XI double KO (ApoE/FXI DKO) mice do not develop Aβ plaques.

We first asked whether FXI deficiency would inhibit atherogenesis in very old mice, similar to its effects in younger mice (28). The results show that elderly mice develop advanced atherosclerosis, and 30% of the aortic arch is covered with atherosclerotic lesions. This is in accord with previous reports showing the development of advanced atherosclerotic lesions in ApoE KO mice that have been fed a normal diet (39). FXI deficiency reduced the lesion area by 33%, similar to its effect in 42-week-old ApoE KO mice (28). Congenital FXI deficiency or targeting FXI do not cause spontaneous bleeding (40-42), in contrast to targeting factor VIII or factor IX. Furthermore, it was recently shown that inhibition of FXI synthesis by antisense oligonucleotide reduces blood pressure in mice and rats (43). Therefore, it is conceivable to use FXI KO mice to study the effect of the coagulation pathway on pathological changes in the brain in elderly mice.

As we used very old ApoE KO mice, we anticipated that these mice would develop pathological changes compatible with AD, including senile Aβ plaques. Unexpectedly, no lesions compatible with AD (i.e., Aβ plaques, brain atrophy, increased glial cells or increased levels of cortex inflammatory cytokines, such as IL-6 and TNF-α) were detected

in the ApoE KO mice or in the FXI-deficient mice. There could be several explanations for such findings, which may all relate to ApoE's role in the brain. In contrast to ApoE4 transgenic mice that clearly develop the typical symptoms of AD, the effect of ApoE deficiency on pathological changes compatible with AD is still unknown. Several studies show that ApoE KO mice are associated with an increased risk of developing AD-related pathologies, that is, memory deficit (20), tau phosphorylation (19,26), a leaky blood-brain barrier (BBB) (24), higher levels of protein oxidation (18), age-dependent synaptic loss (23) and even Aβ deposits (26). In addition, a recent study shows synaptic loss and dysfunction in mice that express ApoE in peripheral tissues, but that have severely reduced ApoE in the brain (22). Notably, no pathologic brain changes were observed in 9-month-old ApoE-deficient mice (25). Bales *et al* (17) claimed that the ApoE KO mouse is not a suitable model to study AD, since ApoE facilitates Aβ deposition, while completely ablation of ApoE, decreased cerebral Aβ sedimentation. It is important to note that Bales *et al* demonstrated their findings in mice aged 6-11 months and studied only Aβ deposition in the brain, while we studied 15-month-old mice and also investigated atherosclerosis-related pathologies in the brain. Taken together, ApoE KO and ApoE/FXI DKO cannot serve as a model to study AD or pathologic brain changes related to atherosclerosis. It appears that there is a dichotomy between the brain and peripheral blood vessels. The mechanism by which ApoE KO protects against brain pathology should be further studied as it may prove helpful for future treatment for senile dementia. Yet, targeting FXI can still serve as an important therapy to attenuate atherosclerosis. As such, it is conceivably that elderly patients might benefit from FXI target therapy to reduce the process of peripheral atherosclerosis, though more studies are needed.

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