Apolipoprotein-E modulates the cytotoxic effect of *B*-amyloid on rat brain endothelium in an isoform-dependent specific manner

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Abstract. Several studies support the hypothesis that apolipoprotein-E (ApoE) acts as a pathological chaperone protein that promotes the ß-plated sheet conformation of ß-amyloid (AB) peptides into amyloid fibers. In vitro evidence is also available that ApoE inhibits the neurotoxic effect of Aß in an allele-specific manner ($E2 \ge E3 > E4$). We have recently shown that Aß peptides exert a time- and concentration-dependent toxic effect on rat neuromicrovascular endothelial cells (NECs), and this study aimed to ascertain whether ApoE isoforms are able to modulate this effect. ApoE2 and ApoE4 decreased and increased, respectively, the cytotoxic effect of AB(1-40) and AB(1-42) on NECs, as evaluated by their survival and viability rates. The toxic effect of both AB peptides and ApoE4 was associated with the rise in the necrosis rate of NECs within a 24-h incubation period. Moreover, ApoE2 prevented and ApoE4 magnified the inhibitory effect of AB on the capability of NECs cultured on Matrigel to form a capillarylike network. The opposite effects of ApoE isoforms could be due to their different interactions with the C-terminal domain of AB. ApoE2, at variance with ApoE4, is thought to form sodium dodecyl sulphate-stable complexes with Aß and, as a consequence, it could block the interactions of the non-fibrillar Aß peptide with the plasma membrane, Aß peptide aggregation and the ensuing cytotoxicity. Collectively, our findings confirm the view that ApoE plays a relevant role in the pathogenesis of Alzheimer's disease.

Introduction

It is current knowledge that Alzheimer's disease (AD) is associated with brain vascular and neuronal damage, involving the formation of intracellular neurofibrillar tangles (NFT) and

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the accumulation of β -amyloid (A β) peptides in extracellular plaques (1-4). Epidemiological and biochemical observations suggest that endogenous factors, such as apolipoprotein-E (ApoE), affect the rate of A β formation and could play a relevant role in the pathogenesis of AD (5,6). ApoE exists in three isoforms, E2, E3 and E4, which are the products of three alleles (ϵ 2, ϵ 3 and ϵ 4) at a single locus (7). The ApoE genotype is thought to be a susceptibility factor for AD, with the ϵ 4 and ϵ 2 alleles increasing and decreasing the risk of developing AD, respectively. Moreover, ApoE4 and ApoE2 isoform frequency was found to be significantly higher and lower in AD patients than in healthy humans (8).

Evidence has been provided that the ApoE4 isoform is connected with the development of the neuropathological features of AD: senile plaques (SP) and NFT. In fact, it has been shown that a direct interaction between ApoE and Aß peptides affects the *in vitro* formation and growth of amyloid plaques (9-12). In particular, ApoE4 seems to have an elevated affinity for Aß binding, favoring its deposition in SP (13-16). *In vitro* studies also suggest that ApoE modulates Aß peptideinduced neurotoxicity in an isoform-specific manner (17-19). It has been demonstrated that the ApoE2 isoform protects neurones, whereas the ApoE4 isoform is ineffective (20).

In the frame of our current studies on AD and neurodegenerative dementia (8,21), we observed that AB peptides are toxic for rat neuromicrovascular endothelial cells (NECs) in a time- and concentration-dependent manner. AB peptides were found to induce NEC death within 3 h, and electron microscopy evidenced morphological signs of cell degeneration after 24-h exposure (22), caspase-8 activation and oxidative stress being involved in this toxic effect (23). It, therefore, appeared worthwhile to investigate whether ApoE isoforms exert a specific effect on AB(1-40)- and AB(1-42)-induced cytotoxicity on rat NECs.

Materials and methods

Animals and reagents. Sprague-Dawley male rats (350-400 g body weight) were purchased from Charles-River (Como, Italy), and the experiment protocol was approved by the local Ethics Committee for Animal Studies. Aß peptides and ApoE isoforms were obtained from Calbiochem (La Jolla, CA). Endothelial cell growth medium MV2 was provided by PromoCell (Heidelberg, Germany), and Matrigel by Becton-

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Dickinson Labware (Bedford, MA). All other chemicals and reagents were obtained from Sigma-Aldrich Corp. (St. Louis, MO).

NEC cultures. Rats were decapitated, and their brains were promptly removed. NECs were isolated and cultured according to the method of Abbot *et al* (24) with few modifications (25,26). NECs from the 3rd to the 4th passage were plated (1.5x10⁴ cells/cm²) on fibronectin-coated Petri dishes, and cultured in MV2 medium, as previously described (25). After 24 h of culture, the medium was replaced with a fresh one containing AB(1-40) or AB(1-42) (10⁻⁷ M), alone and in the presence of ApoE2 or ApoE4 (0.2 μ g/ml). NECs were cultured for another 3, 24 or 48 h. Cell survival was evaluated by trypan blue exclusion, by counting NECs which did not internalize the dye.

Redox activity. At the end of the incubation, NEC redox activity was measured using the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI), a colorimetric method for determining the number of viable cells in proliferation, cytotoxicity or chemosensitivity assays. The assay is based on the ability of living cells to convert a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] to purple formazan (27). Briefly, MTS dye was added to the culture medium (0.5 mg/ml), dishes were incubated for 90 min, and the quantity of formazan was measured by recording absorbance at a 490-nm wavelength using a microplate autoreader, EL-13 (Bio-Tek Instruments, Winooski, VT). The amount of 490-nm absorbance is directly proportional to the number of living cells in culture.

Cell necrosis. Cell death (necrosis) was determined, by measuring the release of lactate dehydrogenase (LDH), using an LDH-cytotoxicity detection kit (Roche, Mannheim, Germany). The activity of LDH was determined by measuring its ability to reduce tetrazolium salt to formazan in the presence of NAD⁺ and diaphorase. Formazan production was assayed by measuring absorbance at a 490-nm wavelength in a microplate autoreader, EL-13.

In vitro angiogenesis. Matrigel was thawed on ice overnight and spread evenly over each well (50 μ l) of a 24-well plate. The plates were incubated for 30 min at 37°C to allow Matrigel to gel, and NECs were seeded (2.5x10⁴ cells/cm²) and cultured in MV2 medium, containing Aß peptides and ApoEs, as described above. After 24 h of incubation at 37°C, cultures were photographed (5 fields for each well: the four quadrants and the center) at a magnification of x5. Image analysis was carried out as previously described (22,25,28) and the following parameters were estimated: percent area covered by NECs, total length of NEC network per field, and number of meshes and branching points per fields.

Statistics. Results were expressed as percent change from baseline value and were the mean \pm SEM of four separate experiments. Statistical analysis was performed by ANOVA, followed by the Student-Newman-Keuls t-test as post-hoc test.



Figure 1. Effects of ApoE2 (upper panel) and ApoE4 (lower panel) on the survival of NECs cultured in the presence of Aß peptides, as measured by trypan blue exclusion assay. Bars are means \pm SEM of four separate experiments. *p<0.05 and **p<0.01 from the respective baseline value; *p<0.05 and **p<0.01 from the respective Aß-peptide value.

Results

Trypan blue exclusion showed that both Aß peptides decreased NEC survival after an incubation period of 1 and 3 h but not 24 or 48 h. Co-treatment with ApoE2 evoked a significant decrease in cell survival at 1 h while, at 3 h, it induced a significant increase with respect to the Aß-peptide values. NEC survival was not affected by ApoE2 co-treatment after 24 h, and only the exposure for 48 h evoked a decrease in this parameter, although the effect was significant only in the case of treatment with Aß(1-42) (Fig. 1, upper panel). Co-treatment with ApoE4 induced a significant decrease in NEC survival, with respect to baseline values after all incubation periods and also with respect to the Aß-peptide values after 24 and 48-h incubation periods (Fig. 1, lower panel).

Redox activity of NECs, as evaluated by MTS-reduction assay, was not affected by A β peptides at 1 h, while it was significantly inhibited by A β (1-42) at 3 h and by both A β peptides at 24 h. Redox activity was restored at 48 h. Co-



Figure 2. Effects of ApoE2 (upper panel) and Apo E4 (lower panel) on the cell survival of NECs cultured in the presence of A β peptides, as measured by MTS-reduction assay. Bars are means ± SEM of four separate experiments. *p<0.05 and **p<0.01 from the respective baseline value; *p<0.05 and **p<0.01 from the respective A β -peptide value.

treatment with ApoE2 significantly lowered redox activity with respect to the baseline value at 3 h. However, at 24 and 48 h, ApoE2 induced an increase in redox activity, which was significant with respect to the A β (1-40) value at 24 h and to the A β (1-42) value at 48 h (Fig. 2, upper panel). MTS reduction was inhibited by co-treatment with ApoE4, the inhibition being significant with respect to the A β (1-42) value at 3 h and to both A β -peptide values at 24 h.

NEC death (necrosis), as assayed by LDH release, was significantly increased after 24-h exposure to both Aß peptides. The presence of ApoE2 prevented Aß peptide-induced necrosis at 24 h but not at 3 and 48 h. ApoE4 induced a significant increase, with respect to both baseline and Aß-peptide values, after an incubation period of 3 h but not of 24 or 48 h (Fig. 3).

NECs cultured on Matrigel spread and aligned with each other to form branching anastomosing tubes that gave rise within 24 h to a meshwork of capillary-like structures (Fig. 4). Quantitative image analysis showed that Aß peptides inhibited the network formation. Co-treatment with ApoE2 not only prevented the Aß peptide-inhibitory effect but also induced a significant increase in the network formation. Co-treatment with ApoE4 significantly downloaded the capillary-like network formation with respect to both baseline and Aß-peptide values (Fig. 5).



Figure 3. Effects of ApoE2 (upper panel) and ApoE4 (lower panel) on the necrosis rate of NECs cultured in the presence of AB peptides, as measured by LDH-release assay. Bars are means \pm SEM of four separate experiments. *p<0.05 and **p<0.01 from the respective baseline value; *p<0.05 and **p<0.01 from the respective AB-peptide value.



Figure 4. Phase contrast micrograph illustrating the arrangement of NECs into a meshwork of capillary-like tubules when cultured on Matrigel for 24 h. Magnification x60.

In all experiments, neither ApoE2 nor ApoE4 *per se* had any significant effect (data not shown).

Discussion

ApoE is found in all main pathological lesions of AD, and the differences in the physiological activity among different



Figure 5. Effects of ApoE2 (upper panels) and ApoE4 (lower panels) on the dimensional [percent area (area) covered by NECs and total length per field (length)] and topological parameters [number of branching points per field (branching) and number of meshes per field (number)] of NECs cultured on Matrigel in the presence of Aß peptides. Bars are means \pm SEM of four separate experiments. *p<0.05 and **p<0.01 from the respective baseline value; *p<0.05 and **p<0.01 from the respective Aß-peptide value.

ApoE isoforms have been considered of great interest (13,14,29,30). *In vitro* observations support the hypothesis that ApoE acts as a pathological chaperone protein, which promotes the β -plated sheet conformation of A β into amyloid fibers, and provides a possible explanation for the association between ApoE4 isoform and AD (31,32). *In vivo* studies, carried out in an AD mouse model, demonstrated ApoE isoform-dependent effects on the amount and anatomical distribution of A β deposits (12,33). The expression of ApoE2 markedly reduces and that of ApoE4 raises hippocampal A β content and A β (1-42) levels (34). *In vitro* studies confirmed the specific effect of ApoE isoform in protecting neuronal and cerebrovascular smooth muscle cells from the toxic action of A β peptides, ApoE2 being more and ApoE4 being less effective (18,20,35).

Our present results demonstrate that ApoE2 has an inhibitory effect, mainly within a 24-h incubation period, on the cytotoxic action of A β peptides on NECs, while ApoE4 has a long-lasting magnifying effect. The survival and viability of these cells are increased and reduced, respectively, by ApoE2 and ApoE4 with respect to A β (1-40) and A β (1-42) values. Moreover, the toxic effect of both A β peptides and ApoE4 appear to be mediated by an increase in the necrosis rate within a 24-h incubation period.

ApoE2 and ApoE3, unlike ApoE4, seem to form sodium dodecyl sulphate-stable complexes with Aß peptides and could mediate Aß-peptide clearance by means of specific membrane receptors, particularly LRP (LDH receptor-related protein). Moreover, ApoE-Aß interaction inhibits the interactions of the non-fibrillar AB with the plasma membrane, Aß aggregation and its subsequent cytotoxicity (18-20,36,37). This hypothesis agrees with the studies of Pillot et al, who demonstrated that the lipid-binding C-terminus of ApoE interacts, in an isoform-specific manner, with Aß peptides (38). In ApoE2 and ApoE3, which possess a Cys residue in the 112 position, Arg⁶¹ forms a salt bridge with Glu¹⁰⁹ so that the two isoforms have a specific three dimensional organization, which allows their C-terminal domain to interact with that of Aß peptides. This domain displays fusogenic properties, which might account for the cytotoxicity of Aß peptides. In fact, their interaction with specific cell membrane phospholipids, e.g. phosphatidylserine (39), may induce destabilization of the cell membrane (40). As a consequence, ApoE2 association with the Aß C-terminal domain, by impairing Aß interaction with the cell membrane, may decrease its fusogenic properties. The substitution Cys/Arg at the 112 position in ApoE4 modifies the conformation of the full-length protein, due to specific interactions between the N- and C-terminal domains, thereby preventing the binding of the C-terminus to Aß peptides (38).

Our results also demonstrate that, in the presence of ApoE2, NECs do not release significant amounts of LDH which, on the contrary, is increased by Aß-peptide exposure. This ApoE2 protective effect could be due to the ability of this isoform to interact with the C-terminal domain of Aß peptides. In contrast, ApoE4 exerts a necrosis effect on NECs within 24 h of exposure, followed by NEC recovery at 48 h. These data are in keeping with other studies on neuroblastoma cells, which showed that ApoE4 and AB(1-42) modulate the activation of glycogen synthase kinase-3ß (GSK-3ß), an enzyme involved in cell death and tau abnormal phosphorylation, in a biphasic

manner. In fact, ApoE4 first activates GSK-3ß via both Ca²⁺dependent and -independent mechanisms and then inhibits it, probably via the activation of kinases that subsequently inactivate GSK-3ß by phosphorylation (41,42). The hypothesis has been advanced that a defect in GSK-3ß regulation may contribute to AD development (42).

Matrigel assay confirms the ApoE-isoform specific effect on Aß peptide cytotoxicity; co-treatment with ApoE2 induces an increase in the formation of the meshwork, while cotreatment with ApoE4 magnifies the inhibitory effect of Aß peptides. This last finding agrees with the recently demonstrated *in vitro* and *in vivo* antiangiogenic action of ApoE4 (43).

In light of the present study, we conclude that ApoE plays a relevant role in the pathogenesis of AD, since ApoE2 lowers and ApoE4 increases the cytotoxic effects of AB peptides on vascular brain endothelium.

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