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Caspase-8 activation and oxidative stress are involved in the cytotoxic effect of ß-amyloid on rat brain microvascular endothelial cells

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Abstract. Several studies have demonstrated that cerebrovascular dysfunction and damage play a significant role in the pathogenesis of Alzheimer disease (AD). In fact, ß-amyloid peptides (Aßs), the major component of the senile plaques and cerebrovascular amyloid deposits in AD, were shown to be cytotoxic to endothelial cells. We have recently observed that Aßs exert a toxic effect on neuromicrovascular endothelial cells (NECs) in a time- and concentration-dependent manner, apoptosis playing a pivotal role in this process. Hence, it seemed worthwhile to investigate the Aß-mediated apoptosis mechanism in NECs. Aßs were found to induce, after a short incubation period, apoptosis throughout caspase-8 activation. Moreover, Aßs elicited a highly significant ($p<0.001$) increase in superoxide dismutase (SOD) levels after a 3-h exposure period, while SOD concentration was not affected after a 24-h incubation. The time-dependent increase in SOD concentration is probably correlated with the production of an excess of reactive oxygen species. Collectively, our findings allow us to conclude that: i) Aßs may induce apoptosis via the activation of caspase-8, presumably by cross-linking and activating receptors of the death-receptor family; ii) oxidative stress is possibly involved in the Aß-induced cytotoxic effect; and iii) these two mechanisms do not act sequentially but, probably, are independent of each other.

Introduction

Evidence has been provided that, ß-amyloid peptides (Aßs) may accumulate not only in the brain parenchyma but also in the cerebral blood vessels (1,2) and that this is a major cause of hemorrhagic and ischemic strokes in elderly patients with or without Alzheimer disease (AD). *In vitro* studies demonstrated that Aßs exert, in a time- and concentration-dependent manner, a marked toxic effect on cerebral endothelial cells (ECs) (3-5). Moreover, Aßs have been shown to be neurotoxic, inducing cell death and oxidative stress in cultured neurons (6,7). Although the mechanism by which extracellular Aßs trigger endothelial and neuronal death has not yet been fully elucidated, the predominant hypothesis suggests the involvement of apoptotic cell death (8). Loo *et al* (9) reported that the exposure of cultured neurons to Aßs induces degeneration and cell death, which seems to occur via an apoptotic pathway. Neuronal cultures exposed to Aßs degenerate, exhibit nuclear chromatin condensation and plasma membrane blebbing. We have previously shown that also neuromicrovascular ECs (NECs) treated with Aßs undergo apoptosis (5).

At present, two major pathways of apoptosis are recognized: the death-receptor pathway in which caspase-8 plays a pivotal initiator role and the mitochondrial pathway involving oxidative stress and activation of caspase-9 (10). Caspase-8 is typically activated via ligand binding to death-receptors such as TNF-R1, Fas and others. Activation of caspase-8 triggers a proteolytic cascade, resulting in the activation of effector caspases, including caspase-3. In contrast, caspase-9 is activated through a complex pathway of mitochondrial dysfunction, mitochondrial-related oxidative stress and cytochrome c release, which eventually results in the activation of caspase-3 (11).

Recent studies provided evidence of the activation of caspase-8 in the AD brain, and suggested that this activation may occur through the stimulation of receptors in the death-receptor pathway (12). Moreover, *in vitro* investigations carried out on neurons and cerebral ECs, suggested that the Aß-induced apoptotic pathway requires caspase-8 activity (13,14). Mitochondrial dysfunction and mtDNA damage was also detected in ECs, probably caused by oxidative stress (14). However, it remains to be settled whether the two mechanisms mediating the Aß-induced EC death, i.e. caspase activation and oxidative stress, act sequentially or independently of each other.

In the frame of our current studies on AD and neurodegenerative dementia (5,15,16), we sought to investigate the role of caspase activation and oxidative stress in Aß-mediated NEC apoptosis.

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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(1-40) and Aß(1-42), alkaline phosphatase conjugate anti-IgG, 5-bromo-4-chloro-3-indolyl phosphatase (BCIP) and p-nitroblue tetrazolium chloride (NBT) were provided by Calbiochem (La Jolla, CA). EC growth medium MV2 was obtained from PromoCell (Heidelberg, Germany) and the BCA protein assay kit was from Pierce (Rockford, IL). Polyvinylidene fluoride membrane (PVDF) was purchased from Gelman Science (Ann Arbor, MI) and benzyloxycarbonyl-IEDT-fluoromethylketone (Z-IEDT-FMK), rabbit anti-caspase-8 polyclonal antibody and a CaspaTag™ Caspase-8 *In Situ* assay kit were from Chemicon International (Temecula, CA). Bovine serum albumine (BSA), phosphatate-buffered saline (PBS) and all other chemicals and reagents were provided by Sigma-Aldrich Corp. (St. Louis, MO).

NEC culture and treatment. Rats were decapitated, and the brain was promptly removed. NECs were isolated and cultured according to the method of Abbot *et al* (17), with few modifications (5,18). NECs from the 3rd and 4th passage were plated ($1.5x10^4$ cells/cm^2) on fibronectin-coated Petri dishes, and cultured in MV2 medium, as previously described (19). After 24 h of culture, medium was replaced with a fresh one containing Aß(1-40) or Aß(1-42) (10^{-7} M).

Western blotting. NECs were treated for 1, 3, or 24 h, and then were washed with PBS and lysed for 30 min on ice using cell lysis buffer (50 mM Tris-HCl, 2 mM EDTA, aprotinin 10 μg/ml, leupeptin 10 μg/ml, phenylmethylsulfonylfluoride 1 mM). The protein concentration was measured using the BCA protein assay kit. Total 25 μg proteins were loaded onto a 12% sodium dodecyl sulfate-polyacrylamide electrophoresis gel and transferred to PVDF membrane. The membrane was then blocked overnight at 4˚C with Tris-buffered saline solution containing 2% BSA, followed by incubation with rabbit anti-caspase-8 polyclonal antibody (1:200 dilution). The membrane was then washed with the blocking solution prior to incubation with alkaline phosphatase conjugate anti-IgG (1:4000 dilution). Blots were developed using a solution containing 0.56 mM BCIP and 0.48 mM NBT in Tris-buffered saline solution (pH 9.5).

In vitro assay of caspase-8 activity. Caspase-8 activity was examined using a CaspaTag™ Caspase-8 *In Situ* assay kit (Chemicon International). The kit uses a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase-8 (FAM-LETD-FMK), which produces a green fluorescence. Briefly, after a 3-h incubation period, fluorochome caspase-8 inhibitor reagent was added to the culture medium. Cells were observed under a fluorescence microscope using a bandpass filter (excitation 490 nm, emission 520 nm wavelength) to view the green fluorescence of caspase-8 positive cells.

Cell survival in the presence of caspase-8 inhibitor. For experiments using caspase-8 inhibitor, NECs ($2.5x10^4$ cells/cm^2) plated in 24-well dishes were pretreated with 20 μM Z-IETD-FMK for 1 h, followed by 10^{-7} M Aß peptides for up to 24 h. Cell survival was evaluated by trypan blue exclusion, by counting NECs which did not internalize the dye.

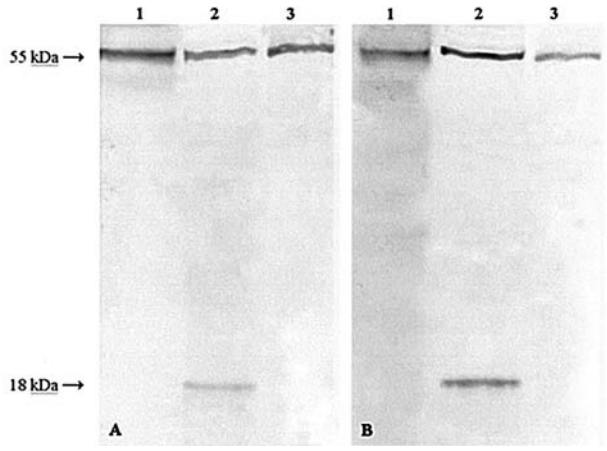

Figure 1. Western blot analysis showing caspase-8 activation in cultured rat NECs, after 1-h (lane 1), 3-h (lane 2) and 24-h incubation (lane 3) with Aß(1-40) (A) and Aß(1-42) (B).

Superoxide dismutase (SOD) assay. The superoxide radical is revealed by a reaction with luminol (5-amino-2,3-dihydro-1,4-ftalazinedione) which is oxidized in 3-amino-ftalic acid emitting light (maximum emission at 425 nm wavelength) measured using a Lumicon Hamilton Luminometer (20). The presence of superoxide dismutase (SOD) in the sample determines the subtraction of radicals through dismutation. At the end of the incubation time, cells were washed with PBS and lysed on ice by cell lysis buffer (0.5% v/v Triton X-100 in 0.154 M carbonate buffer pH 10.2). Two μl of xantine oxidase (1.35 mg/ml), 78 μl of EDTA (0.3 M) and luminol (0.03 mM) in carbonate buffer (0.154 M, pH 10.2), and 80 μl of hypoxantine (30 μM) were mixed with 80 μl of sample. The protein concentration was measured using the BCA protein assay kit. The calibration curve was constructed by using SOD from bovine erythrocyte (1 μg/ml) (ε=10300/cm^{-1}/mol^{-1}).

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

Results

Western blot assay performed on cell lysates prepared from NECs after 1-, 3- and 24-h treatment with Aß(1-40) and Aß(1-42) showed a time-dependent proteolytic cleavage of caspase-8. After a 1-h incubation period, a 55-kDa protein band was detected, corresponding to inactive procaspase-8. Two bands with molecular sizes of 55 and 18 kDa were detected after 3 h of incubation, the 18-kDa protein representing the fully processed active enzyme resulting from two-step proteolysis. After 24-h treatment, only the 55-kDa protein band was detected (Fig. 1).

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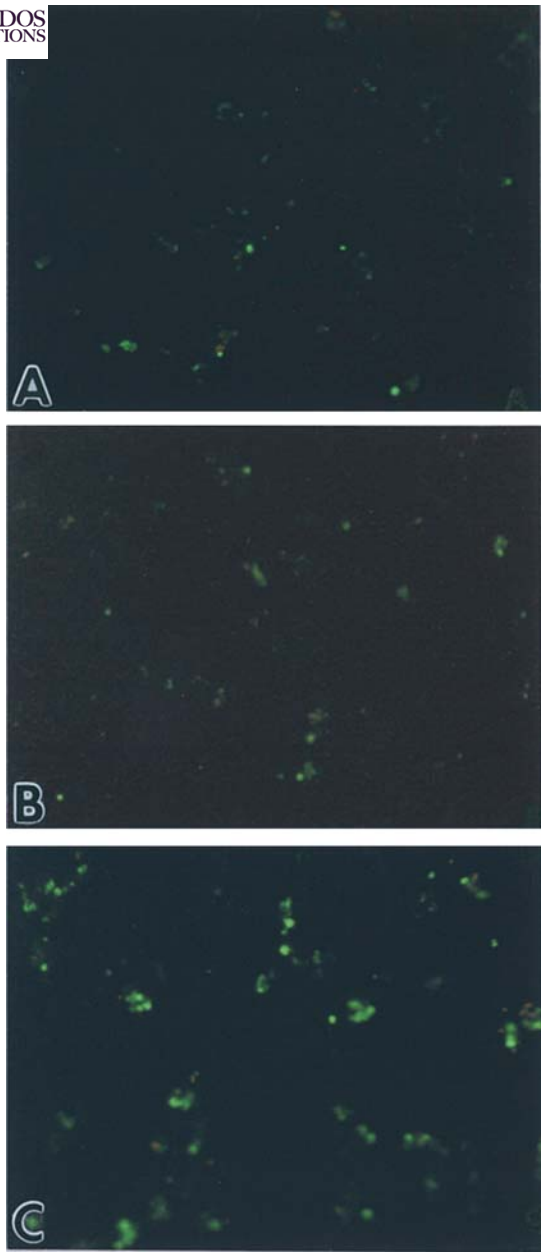

Figure 2. CaspaTag caspase-8-positive rat NECs in control cultures (A), and after incubation for 3 h with Aß(1-40) (B) or Aß(1-42) (C).

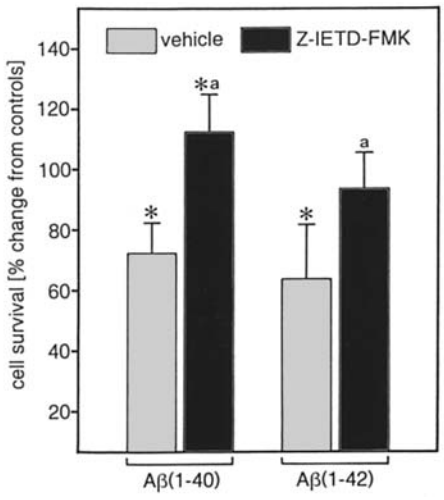

Figure 3. Trypan blue exclusion assay showing the effect of Z-IETD-FMK on the survival of cultured rat NECs treated for 3 h with Aß(1-40) or Aß(1-42). Bars are means ± SEM of four separate experiments. $^{*}p<0.05$ from the respective control group; $^{a}p<0.05$ from the respective vehicle group.

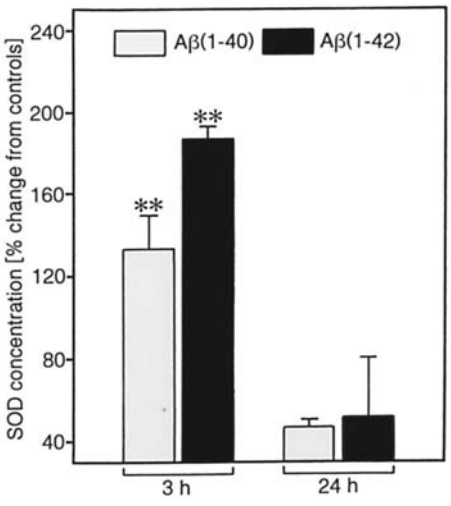

Figure 4. Luminol assay showing the effects of Aß(1-40) and Aß(1-42) on SOD concentration in cultured rat NECs. Bars are means ± SEM of four separate experiments. $^{**}p<0.001$ from the respective control group.

CaspaTag Caspase-8 *In Situ* assay confirmed the activation of caspase-8 after 3 h of Aß(1-40) and Aß(1-42) treatment. As shown by fluorescence microscopy, NEC cultures treated with Aßs for 3 h displayed the presence of activated caspase-8. Moreover, the isoform, Aß(1-42), appeared to have a more toxic effect than Aß(1-40) (Fig. 2).

Trypan blue exclusion showed that, after a 3-h incubation period, both Aßs decreased NEC survival. Pretreatment with the caspase-8 specific and irreversible inhibitor, Z-IETD-FMK, significantly ($p<0.05$) protected NECs from Aß-induced cell death (Fig. 3).

In NEC cultures, both Aßs induced a highly significant ($p<0.001$) increase in SOD levels after a 3-h exposure period, Aß(1-42) being more effective than Aß(1-40) ($p<0.001$). SOD concentration levels were not affected by either Aß after a 24-h incubation period (Fig. 4).

Discussion

Aßs, the major component of senile plaques and of cerebrovascular amyloid deposits in Alzheimer disease (AD), have been shown to be cytotoxic to ECs (3,4,21-23). Specifically, amyloid fractions purified from AD brain inhibit EC replication *in vitro* and, therefore, could alter the ability of vessels to repair and regenerate after injury (24). Moreover, Aßs are directly toxic either to peripheral and cerebral vascular endothelium (3,4,25). Xu *et al* (14) confirmed the cytotoxic effect of Aßs on bovine and murine cerebral ECs. Their findings indicate that Aß-mediated EC death is an apoptotic process that is characterized by caspase activation, mitochondrial dysfunction and increased oxidative stress. We recently demonstrated that Aßs are toxic for rat NECs in a time- and concentration-dependent manner. Aßs were found to induce NEC death within 3 h and electron microscopy evidenced morphological

signs of cell degeneration after 24-h exposure. Moreover, our findings indicate that apoptosis seems to play a pivotal role in Aß-induced NEC death (5).

Our results show the activation of caspase-8 in NEC cultures treated with Aßs, as already observed in neuronal cultures (13). Particularly, Western blot analysis demonstrates the expression of the active form of caspase-8 within 3 h while, after 24 h, only the procaspase-8 form is expressed. Our findings obtained using CaspaTag assay and the selective caspase-8 inhibitor, Z-IETD-FMK, confirm those of the Western blot analysis and suggest that caspase-8 is likely to be the primary initiator caspase involved in Aß-mediated apoptosis of NECs. Our results accord well with that of Rohn *et al* (12) who, by means of *in vivo* immunohistochemical analysis, provided evidence for the activation of caspase-8 in the brain neurons of AD patients (12). Moreover, quantitative and statistically significant differences in the activation of caspase-8 in the peripheral blood mononuclear cells of AD patients have been recently observed (26). Our present findings support the possibility that Aß may induce apoptosis through the activation of caspase-8, presumably by cross-linking and activating receptors of the death-receptor family (12-14).

An important role in cell death seems to be played by oxidative stress. Thomas *et al* (3) demonstrated that Aß interacts with vascular EC to produce an ROS excess, which alters EC structure and function. In addition, free radical scavengers, such as SOD, have been shown to be protective against Aß cytotoxicity (3,28,29). We found that Aßs significantly increase SOD concentration as early as 3 h after incubation. The time-dependent increase of SOD concentration is probably correlated with production of an excess of ROS. Hence, this observation suggests a possible involvement of oxidative stress in the Aß-induced cytotoxic effect.

It seems reasonable to advance the hypothesis that the apoptotic effect induced by Aßs, at a concentration of 10^{-7} M, on rat NECs occurs through two different pathways: caspase-8 activation and increase of oxidative stress. Based on our findings and considering that both pathways are activated by Aßs after a short incubation time (3 h) and that, after a 24-h incubation period, SOD concentration is no longer affected by Aßs, we assume that the two mechanisms do not act sequentially but are probably independent of each other.

The present observations support our previous data demonstrating that the maximum cytotoxic effect of Aßs on NECs occurs within 3 h of exposure, followed by an NEC recovery at 24 h. Moreover, they are in agreement with other studies which showed that Aß(1-42) modulates the activation of glycogen synthase kinase-3ß (GSK-3ß), an enzyme involved in cell apoptosis regulation and tau abnormal phosphorylation, in a biphasic manner (29,30): Aß first activates GSK-3ß via both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms and subsequently inhibits it, probably via the activation of kinases that inactivate GSK-3ß by phosphorylation.

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