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Toxicity of Various Amyloid β Peptide Species in Cultured Human Blood–Brain Barrier Endothelial Cells: Increased Toxicity of Dutch-Type Mutant

Patricia B. Eisenhauer,[1,2] Robin J. Johnson,[3,4] John M. Wells,[1,2] Theresa A. Davies,[3] and Richard E. Fine[1–3]★

[1]Geriatric Research Center, Bedford, Massachusetts
[2]Department of Neurology, Boston University School of Medicine, Boston, Massachusetts
[3]Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts
[4]Department of Anatomy and Neurobiology, Boston University School of Medicine, Boston, Massachusetts

The amyloid β peptide (Aβ) is the major component of the neuritic and cerebrovascular amyloid plaques that are one of the characteristic features of Alzheimer's disease (AD). This peptide has been shown to be toxic to several relevant cell types, including neurons, cerebrovascular smooth muscle cells, and endothelial cells. We have studied the toxic effects of both soluble and aggregated species of $A\beta_{1-40}$ and the mutation $A\beta_{1-40}Glu \rightarrow Gln_{22}$, which is the major species deposited in the cerebrovascular blood vessels of victims of hereditary cerebral hemorrhage with amyloidosis, Dutch type. We find that aggregates of both peptides, as well as of $A\beta_{1-42}$ and $A\beta_{25-35}$, are toxic to cultured human cerebrovascular endothelial cells (hBEC) obtained from the brain of a victim of AD (at doses lower than those that are toxic to CNS neurons or leptomeningeal smooth muscle cells). Soluble $A\beta_{1-40}$ Gln_{22} is equally toxic to hBEC, whereas wild-type $A\beta_{1-40}$ is toxic only at higher doses. This toxicity is seen at the lowest dose of $A\beta_{1-40}$ Gln_{22} used, 20 nM. The soluble $A\beta_{1-40}Gln_{22}$ aggregates on the surface of the cells, in contrast to $A\beta_{1-40}$, and its toxicity can be blocked both by an inhibitor of free radical formation and by Congo red, which inhibits amyloid fibril formation. We discuss the possibility that the enhanced toxicity of $A\beta_{1-40}Gln_{22}$ is mediated by a Aβ receptor on the endothelial cells. J. Neurosci. Res. 60:804–810, 2000.

Key words: Aβ receptor; oxidative free radicals; cerebral amyloid angiopathy; hereditary cerebral hemorrhage with amyloidosis, Dutch type; Alzheimer's disease

Amyloid β-peptide (Aβ) a major component of neuritic plaques, is also found deposited in the vessel walls of the cerebrovascular system, causing cerebral amyloid angiopathy (CAA; Buee et al., 1994; Vinters et al., 1994). CAA is a common pathological feature of Alzheimer's disease (AD) and related disorders including Down's syndrome and hereditary cerebral hemorrhage with amyloidosis, Dutch type (HCHWAD). The Aβ that is deposited in the cerebral blood vessels is a proteolytic fragment of the much larger precursor protein, the β-amyloid protein (βAPP). βAPP is a transmembrane glycoprotein that is produced by a variety of cells, including human blood–brain barrier (BBB) endothelial cells (hBECs) and cerebrovascular smooth muscle cells (Wisniewski et al., 1994; Wells et al., 1995; Davis-Salinas and Van Nostrand 1995) as well as by neurons and all forms of glia (for review see Selkoe, 1994; Yankner, 1996).

Individuals with the Dutch mutation have been shown to possess a point mutation in their βAPP gene that results in a Glu→Gln substitution at position 22 in the Aβ domain (Levy et al., 1990). One possible explanation for the pathology of HCHWAD is that Aβ Gln_{22} selectively damages the vessels, resulting in cerebral hemorrhaging and rupturing of vessels leading to disruption of the BBB.

It has recently been shown that $A\beta_{25-35}$ is toxic to cultured bovine pulmonary artery-derived endothelial cells (Blanc et al., 1997). This toxicity is alleviated by both antioxidants and Ca^{2+} channel blockers, indicating that both free radicals and Ca^{2+} homeostatic disregulation are involved in Aβ toxicity. Also, very rapid effects on vasoactivity, including free radical production causing increased vascular tone and endothelial damage, are produced by $A\beta_{1-40}$ addition (Thomas et al., 1996). More recently it has been shown that fresh $A\beta_{1-42}$ and $A\beta_{25-35}$ are very toxic to human endothelial cells, whereas $A\beta_{1-40}$ did not produce significant toxicity (Suo et al., 1997). On the other hand, aged $A\beta_{1-40}$ did produce significant tox-

★Correspondence to: Dr. Richard E. Fine, Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118.
E-mail: fine@med-biochem.bu.edu

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icity. Again both antioxidants and Ca^{2+} channel blockers prevented toxicity.

Recently a novel multivalent $A\beta_{1-40}$-bovine serum albumin conjugate was shown to be toxic to bovine cerebral endothelial cells when added to the cultures at 50–100 nM (Huang et al., 1998). In this report we describe the effects of soluble and aggregated Aβ peptides of various sizes as well as the $A\beta_{1-40}G1u\rightarrow G1n_{22}$ peptide on cultured human BBB endothelial cells. We demonstrate that the enhanced toxicity of the mutant peptide correlates with its association with the cell surface. This finding, together with the pronounced toxicity of the peptide at low nanomolar concentration, suggests that a receptor is involved.

MATERIALS AND METHODS

Cell Culture

Primary cultures of hBECs were isolated from cortical gray matter as previously described (Wells et al., 1995; Eisenhauer et al., 1998). The hBECs used in the study were from a 62-year-old male (post mortem interval ~5 hr) with neuropathologically confirmed AD. Briefly, the brain obtained at autopsy was minced, and the capillary fragments were digested in Hank's balanced salt solution containing 0.2% collagenase and 0.008% trypsin for 1 hr at 37°C. The hBEC were plated onto rat tail collagen-ProNectin F (Protein Polymer Technologies, Inc., San Diego, CA)-coated dishes in medium consisting of 50% minimum essential medium containing 10% plasma derived horse serum, and 50% astrocyte conditioned medium (Rubin et. al., 1991) and supplemented with 1.25 ng/ml bFG, 25 U/ml penicillin, 25 μg/ml streptomycin, and 0.25 μg/ml fungizone. The resulting hBEC were over 95% positive for factor VIII antigen.

Peptides and Experimental Treatments

Wild-type $A\beta_{1-40}$, $A\beta_{1-40}Gln_{22}$ containing the mutation associated with AD Dutch Variant, $A\beta_{1-42}$. and $A\beta_{25-35}$ were obtained from Biosource/Quality Controlled Biochemicals, Inc. (Hopkinton, MA). The $A\beta_{1-40}$ and $A\beta_{1-40}Gln_{22}$ lyophilized peptides were dissolved in double-distilled water to 700 μM, further diluted in PBS to 350 μM, and used immediately (soluble form) or after preincubation at 37°C for 5 days to aggregate the peptides. $A\beta_{1-42}$ was prepared by dissolving the peptide in double-distilled water to 350 μM and used immediately (soluble form) or after preincubation at 37°C for 3 days. $A\beta_{25-35}$ was dissolved in double-distilled water to 4.7 mM and either used immediately or incubated overnight at 37°C. Soluble or fibrillar Aβ was added directly to culture medium to the specified concentration; control cultures received the same volume of PBS vehicle. Peptides were added to confluent hBEC cultures and incubated for 2 days at 37°C.

In experiments assessing the inhibition of Aβ toxicity by Congo red, freshly solubilized Aβ peptides (125 μM $A\beta_{1-40}$ and $A\beta_{1-40}Gln_{22}$) were preincubated alone or with Congo red (125 μM or 250 μM) overnight at 37°C, then added to hBEC cultures (final Aβ concentration 20 μM, final Congo red concentrations 20 or 40 μM). Human BEC viability using the MTT assay was determined after 2 days.

The in vitro protective effects of N-tert-butyl-α (2 sulfophenyl) nitrone (SPBN; Aldrich Chemical Co., Milwaukee, WI) were evaluated using the MTT assay. hBEC cultures were preincubated in the absence of presence of 20 mM or 40 mM SPBN prior to the addition of 20 μM freshly solubilized Aβ peptides. Toxicity was assessed 2 days after the addition of the Aβ peptide.

Cytotoxicity Assay

To assess cell viability (Aβ toxicity), we used the MTT assay, which measures the reduction of 3-(4,5-dimethuylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) to a colored formazan derivative via mitochondrial dehydrogenase activity. The MTT assay was performed according to the instructions of the manufacturer (Promega, Madison, WI). In brief, following the 2 day incubation with peptides, MTT was added and incubation was continued for 4hr at 37°C. Solubilization/stop solution was then added, and absorbances were read at 570 nm using an automated plate reader with dual wavelengths. The data are expressed as the percentage of reduction of MTT relative to controls, where controls received PBS vehicle.

Immunocytochemistry

hBEC grown to near confluency on Lab-Tek chamber slides were incubated in the absence or presence of 20 μM wild-type $A\beta_{1-40}$ or $A\beta_{1-40}Gln_{22}$ in serum-containing medium. After 48 hr at 37°C, the cells were rinsed five times with PBS and fixed for 30 min at room temperature in 2% paraformaldehyde. After rinsing five times with PBS, the cells were incubated for 15 min with protein blocker solution (Research Genetics, Huntsville, AL) and then rinsed three times with PBS. Slides were incubated with mAb 6E10 (1:1,000) from Senetek PLC (Maryland Heights, MO) overnight at 4°C, rinsed three times in PBS, then incubated with fluorescein-coupled rabbit anti-mouse IgG (1:40) for 1 hr at 22°C. The slides were rinsed five times with PBS, and immunofluorescence was visualized and photographed using an Olympus BH-2 light microscope.

Statistical Analysis

The statistical significance of the effects of experimental treatments were analyzed using an analysis of variance (ANOVA). Individual treatment groups were analyzed by Scheffe's test for multiple comparisons of means. The analyses were performed using statview (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Initial experiments were designed to determine the toxicity of the various aggregated Aβ peptides on confluent hBEC cultures. Figure 1 shows a dose-dependent increase in toxicity of all Aβ peptides used. Although all Aβ types tested showed significant dosage effects by ANOVA ($P < 0.001$), as determined by Scheffe's post-hoc test ($P < 0.01$), $A\beta_{1-40}Gln_{22}$ was the only peptide that showed significant toxicity at the lowest dose (20 nM). Figure 2 shows the results when the same concentrations of the various soluble Aβ peptides were incubated with the hBEC cultures. The ANOVA showed all types having a dosage effect ($P < 0.005$); however, most strikingly the $A\beta_{1-40}Gln_{22}$ peptide is again toxic

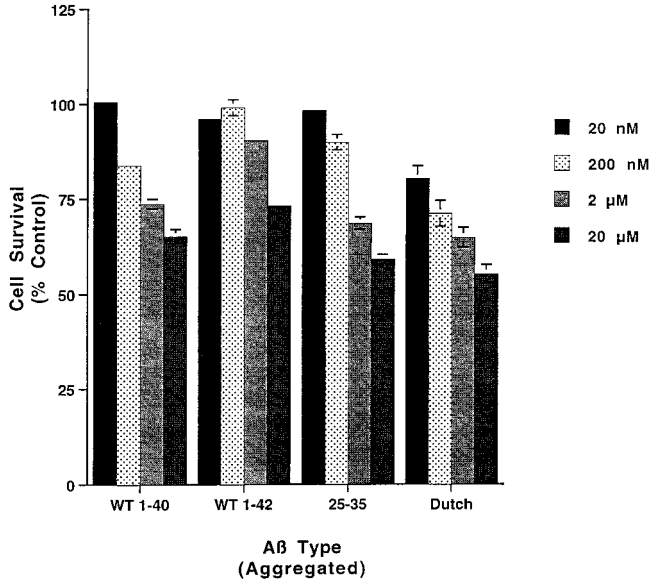

Fig. 1. Effects of aggregated Aβ peptides on cultured hBEC. hBEC were exposed to the indicated forms and doses of Aβ peptides. Two days after exposure, cell viability was determined by the MTT assay. Results are expressed as the percentage of reduction of MTT relative to untreated controls. Each data point represents the mean ± SEM for triplicate cultures from three individual experiments. Vertical bars represent the SEM. In some cases, the SEM is too small to be resolved on the graph. Effects were significant with respect to both dose ($P < 0.001$) and Aβ type ($P < 0.001$) as determined by ANOVA.

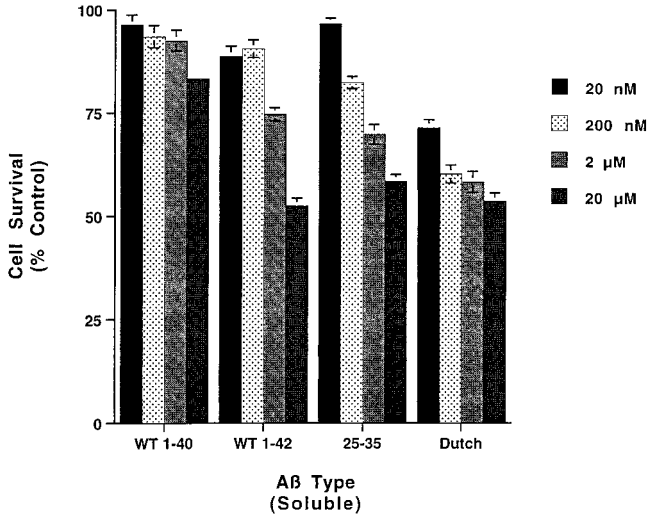

Fig. 2. Effects of soluble Aβ peptides on cultured hBEC. hBEC were exposed to the indicated forms and doses of Aβ peptides. Two days after exposure, cell viability was determined by the MTT assay. Results are expressed as the percentage of reduction of MTT relative to untreated controls. Each data point represents the mean ± SEM for triplicate cultures from three individual experiments. Vertical bars represent the SEM. Again, some SEMs were too small to be resolved. Effects of both dose ($P < 0.005$) and Aβ type ($P < 0.001$) were significant by ANOVA. However, Scheffe's test showed that $A\beta_{1-40}$ was effective only at the highest dose.

at the lowest dose employed (20 nM). Although the ANOVA shows that the wild-type $A\beta_{1-40}$ has a significant dose effect ($P < 0.005$), the Scheffe's test indicates this occurs only at the highest dose tested (20 μM). Incubation with 1 nM $A\beta_{1-40}Gln_{22}$ caused no increase in cell death compared to controls.

$A\beta_{25-35}$ and $A\beta_{1-42}$ did produce significant toxicity at the higher concentrations. The results with the soluble Aβ peptides are similar to those reported by Van Nostrand et al. (1998), in that the $A\beta_{1-40}Gln_{22}$ showed significant toxicity to human leptomeningeal smooth muscle cells (HLSM), whereas wild-type $A\beta_{1-40}$ was not toxic.

This group also demonstrated that the toxicity of $A\beta_{1-40}Gln_{22}$ peptide on HLSM cells correlated with the formation of large Aβ aggregates on the cell surface (Van Nostrand et al., 1998). We carried out a similar experiment using hBEC, and, as Figure 3A demonstrates, we found large Aβ aggregates on the cell surface when these cells were incubated with 20 μM soluble $A\beta_{1-40}Gln_{22}$. In contrast, a few small aggregates were seen using the same concentration of wild-type $A\beta_{1-40}$ (Fig. 3B). When we tested preaggregated $A\beta_{1-40}Gln_{22}$ (Fig. 3E) and $A\beta_{1-40}$ (Fig. 3F) peptides at the same concentration, both formed large cell-associated aggregates (even though the aggregates found with the Dutch peptide were larger and had a bubbly appearance). In all cases areas without cells contained no aggregated material (Fig. 3C,D). In view of the similarities between these findings, we also tested to see whether soluble $A\beta_{1-40}Gln_{22}$ up-regulated βAPP synthesis as was shown to occur in the HLSM (Van Nostrand et al., 1998). In contrast to these findings, we saw no significant increase in either cellular or secreted βAPP (data not shown).

In view of our finding that soluble $A\beta_{1-40}Gln_{22}$ is highly toxic to hBEC, we determined the effect of Congo red, an amyloid fibril-binding dye, on the toxicity of $A\beta_{1-40}Gln_{22}$. Congo red has been reported to block the cell surface fibril assembly of $A\beta_{1-40}Gln_{22}$ on cultured HLSM (Van Nostrand et al., 1998) and to also block the pathologic effects on these cells. Preincubation of soluble $A\beta_{1-40}Gln_{22}$ with Congo red followed by addition to the culture medium (final $A\beta_{1-40}Gln_{22}$ 20 μM, final Congo red concentration 40 μM) resulted in a significant decrease in toxicity (Fig. 4). An identical experiment using $A\beta_{1-40}$ showed a small but not significant decrease in toxicity as well.

Because we found a significant toxic effect of several Aβ peptides on hBEC, we then examined the potential mechanism by which Aβ exerts its effect. Other groups have demonstrated the role of oxidation and the generation of free radicals in Aβ-induced toxicity (Mattson et al., 1993a,b; Behl et al., 1994) We, therefore, examined whether treatment with the free radical spin trap SPBN has a protective effect on $A\beta_{1-40}Gln_{22}$-induced toxicity. Figure 5 shows that SPBN dose-dependently protected hBEC against the toxic effects of $A\beta_{1-40}Gln_{22}$.

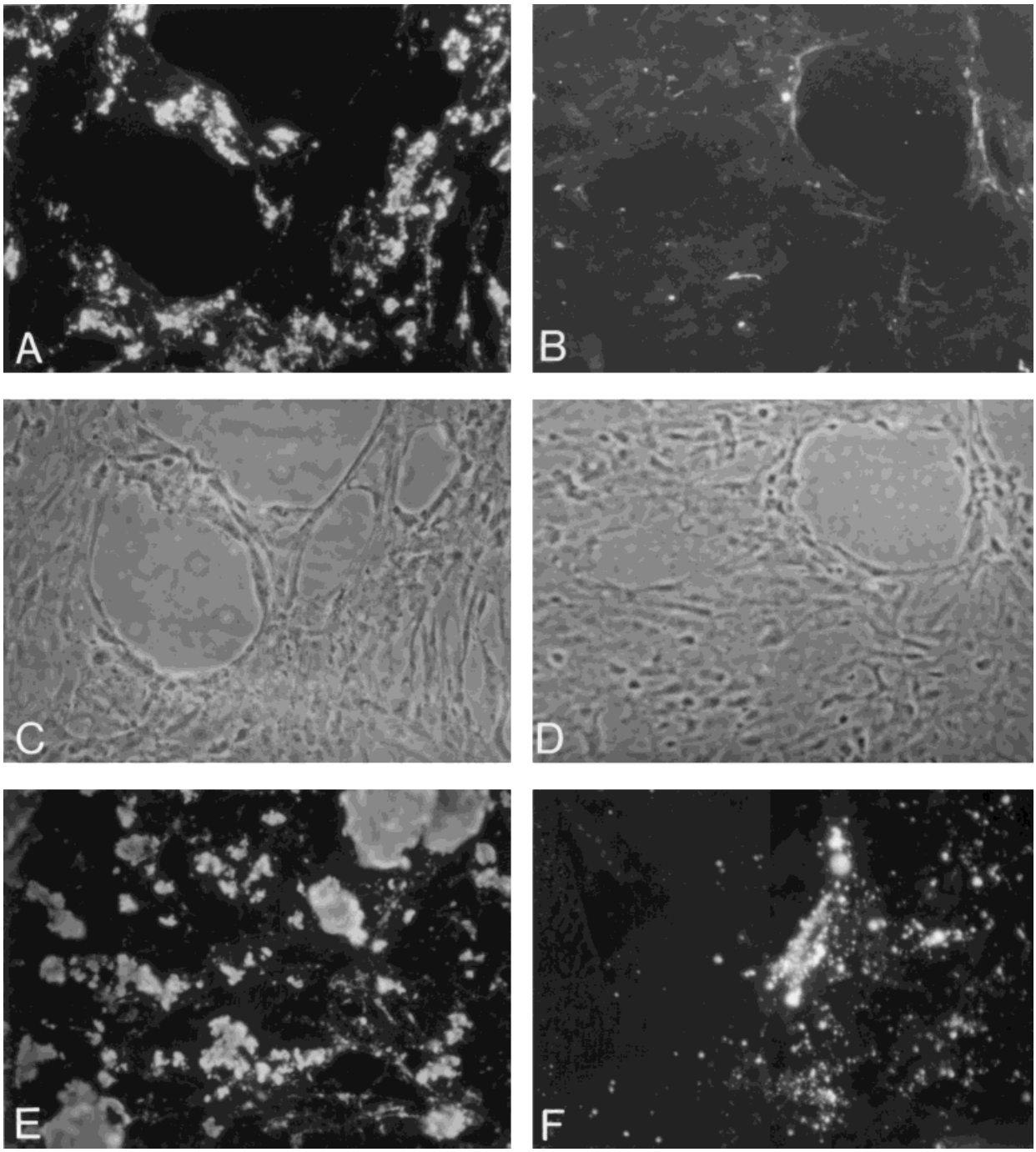

Fig. 3. Aggregation of soluble and aggregated $A\beta_{1-40}$ and $A\beta_{1-40}Gln_{22}$ on the cell surface of hBEC. hBEC cultures were incubated with 20 μM amounts of the Aβ species designated below for 2 days. Cell surface Aβ was detected by immunofluorescence using mAb 6E10 as described in Materials and Methods. The cultures were viewed using an Olympus BH-2 microscope. **A:** Soluble $A\beta_{1-40}Gln_{22}$; fluorescence. **B:** Soluble $A\beta_{1-40}$; fluorescence. **C:** Soluble $A\beta_{1-40}Gln_{22}$; phase contrast. **D:** Soluble $A\beta_{1-40}$; phase contrast. **E:** Aggregated $A\beta_{1-40}$ Gln_{22}; fluorescence. **F:** Aggregated $A\beta_{1-40}$; fluorescence. A–D, ×285. E,F, ×570.

DISCUSSION

Our results indicate that several species of Aβ are toxic to human BECs cultured from the cerebral cortex of an Alzheimer's disease victim. The toxicity is dose-dependent and is first manifested at quite low doses of peptide, 20 nM in the case of $A\beta_{1-40}$ Gln_{22}. This agrees qualitatively with the results of other groups using bovine, rat, and human aorta endothelial cells (Thomas et al.,

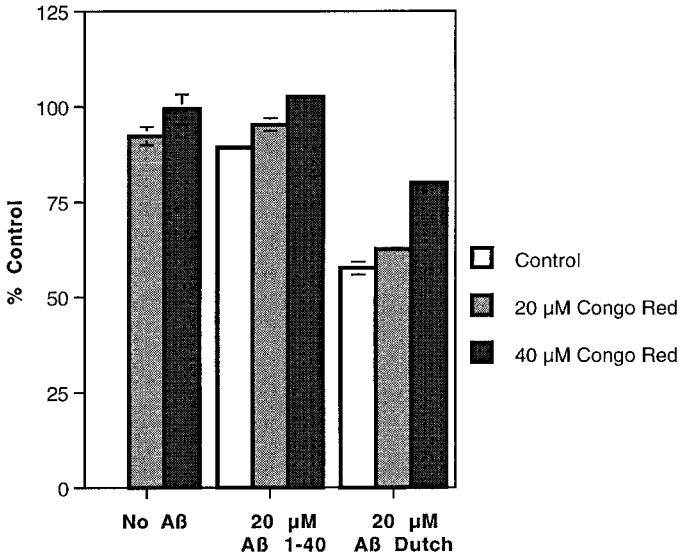

Fig. 4. Inhibition of Aβ toxicity by Congo red. Soluble $A\beta_{1-40}$ and $A\beta_{1-40}Gln_{22}$ (125 μM) were incubated overnight alone or with Congo red (125 or 250 μM) as described in Materials and Methods, then added to hBEC cultures (final Aβ peptide concentration 20 μM, final Congo red concentrations 20 or 40 μM). After a 48 hr incubation, cell viability was assayed using MTT. Values are expressed as percentage of control and represent the mean ± SEM. Some SEMs are too small to be shown on the graph. Data points represent one representative experiment run in triplicate. Similar results were obtained from three different experiments.

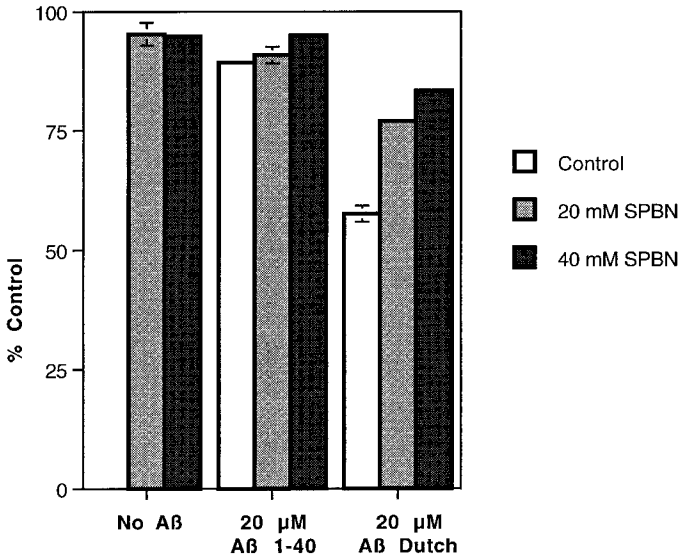

Fig. 5. SPBN reduces the toxicity of Aβ peptides in hBEC. Cells were incubated in the presence of S-PBN at the concentration indicated for 60 min prior to the addition of either 20 μM $A\beta_{1-40}$ or $A\beta_{1-40}Gln_{22}$. After 48 hr incubation, cell viability was assessed using the MTT assay. Values are expressed as percentage of control and represent the mean ± SEM. Some SEMs are too small to be shown on the graph. Data points represent one representative experiment run in triplicate. Similar results were obtained from three different experiments.

1996; Blanc et al., 1997; Suo et al., 1997). From these data it would appear that a reason why the cerebrovasculature is damaged in AD, whereas other vascular beds are spared, might be the production of significant quantities of Aβ only in the brain rather than the increased sensitivity of the BBB endothelial cells themselves.

The most significant finding in our study is that, although both aggregated $A\beta_{1-40}$ and $A\beta_{1-40}Gln_{22}$ are toxic to the cells, only soluble $A\beta_{1-40}Gln_{22}$, which is the major peptide species deposited in the cerebrovasculature of victims with HCHWAD (Castano et al., 1996), is toxic, whereas soluble $A\beta_{1-40}$ is not toxic. Cultured human cerebrovasculature smooth muscle cells also show sensitivity to soluble $A\beta_{1-40}Gln_{22}$, and the $A\beta_{1-40}$ is nontoxic (Davis and Van Nostrand, 1996). One important difference, however, is that neither preaggregated $A\beta_{1-40}$ nor $A\beta_{1-42}$ was toxic to the smooth muscle cells, although they both were toxic to endothelial cells (Davis-Salinas and Van Nostrand 1995).

Our findings also are similar in some respects and differ in certain other respects from those reported by Suo et al. (1997) with human aorta endothelial cells. These cells are considerably more sensitive to $A\beta_{1-42}$ than to $A\beta_{1-40}$, whereas we find that preaggregated $A\beta_{1-40}$ has a significantly greater toxicity on hBEC than does $A\beta_{1-42}$ and that the reverse is true for the soluble peptides. Both cell types are very sensitive to the toxicity of these peptides compared to neurons (Yankner et al., 1990; Mattson et al., 1993b; Pike et al., 1993) or to cerebrovascular smooth muscle cells (Davis and Van Nostrand, 1996). Also, in both types of human endothelial cells, the Aβ-mediated toxicity appears to be mediated via free radicals.

Since both soluble $A\beta_{1-40}$ and $A\beta_{1-40}Gln_{22}$ aggregate at similar rates in solution at the low concentrations used in these experiments (Van Nostrand et al., 1998), it is not likely that the much greater toxicity of the latter species is due solely to a more rapid aggregation of $A\beta_{1-40}Gln_{22}$. However, the fact that we see aggregated $A\beta_{1-40}$ Gln_{22} on the surface of the cell, whereas no aggregated $A\beta_{1-40}$ species are seen (Fig. 3), suggests that there is a binding site on the cells with a much greater affinity for soluble $A\beta_{1-40}Gln_{22}$. This site may serve as a "nucleation" center for the formation of Aβ aggregates and potentially mediates the toxicity of the aggregates. Alternatively, small Aβ oligomers, found to be toxic to mature CNS neurons at nanomolar concentrations (Lambert et al., 1998), may be the toxic species rather than larger aggregates.

In recent years two potential candidates for receptors found on hBECs that bind to Aβ and could mediate its toxicity have been described. The RAGE receptor binds to both soluble and aggregated Aβ species and activates production of a number of cytokines (Yan et al., 1996). It also has a higher affinity for the $A\beta_{1-40}Gln_{22}$ than for $A\beta_{1-40}$.

A second candidate receptor is the TGFβ receptor. Soluble $A\beta_{1-40}$ competes with TGFβ, with an IC_{50} of

approximately 5 μM (Huang et al., 1998). It is also toxic to bovine cerebrovascular endothelial cells, with an IC_{50} of about 20–40 μM. As was mentioned in the Introduction, a multivalent $A\beta_{1-40}$-BSA conjugate is toxic to these cells in the 50–100 nM range. Aβ possesses a four-amino-acid motif ($F_{20}A_{21}F_{22}D_{23}$), which is similar to the WSXD putative motif in various TGF family members and which is essential for receptor binding. Interestingly, based on structural considerations, FAQD, the motif found in the $A\beta_{1-40}Gln_{22}$ peptide, was postulated to have a greater binding affinity for the TGFβ receptor, although no data were presented supporting this claim (Huang et al., 1998). The Aβ-induced toxicity is hypothesized to be caused by interference with the survival-promoting effects of TGFβ on the cells.

Whether or not a "receptor" is involved, it does seem likely, based on our data, that aggregation on the cell surface is required for eliciting the toxicity of Aβ on the cells. One indication is the fact that the toxicity of soluble $A\beta_{1-40}Gln_{22}$ correlates with its aggregation on the surface of the hBECs (Fig. 3). In contrast, the nontoxicity of soluble $A\beta_{1-40}$ correlates with the lack of aggregate formation. Also, both aggregated $A\beta_{1-40}Gln_{22}$ and $A\beta_{1-40}$ were toxic to the hBEC and were associated with the cell surface. Finally, Congo red, a chemical known to interact with soluble Aβ and to block amyloid fibril formation, inhibited the toxicity of $A\beta_{1-40}Gln_{22}$.

That we see large aggregates associated with the cell surface that correlate with the increased toxicity suggests either that the aggregation after binding to a receptor induces a toxic signal such as the induction of apoptosis or necrosis or that the aggregated species of Aβ is a more potent inducer of ROS, which in turn are toxic to the cells. In this regard, our data also agree with numerous other studies indicating that the toxicity of Aβ is produced via the formation of free radicals (for review see Mattson, 1997). SPBN, which is a potent free radical scavenger, inhibits the toxicity produced by $A\beta_{1-40}Gln_{22}$.

In summary, our data indicate that a likely reason for the greater damage to cerebrovascular elements seen in patients with HCHWA-D compared to that seen in AD patients is the much stronger association of soluble $A\beta_{1-40}Gln_{22}$ with the endothelial cell surface. This association and resulting aggregation, potentially mediated via a "receptor," induces free radical-mediated toxicity; it appears that BBB cells are very sensitive to Aβ peptide-mediated toxicity. The fact that both preaggregated Aβ and $A\beta_{1-40}Gln_{22}$ are also toxic to these cells and appear to be cell-associated as well suggests that preformed aggregates of Aβ, potentially produced by adjacent smooth muscle cells, astrocyte, etc., could lead to the ministrokes that contribute to the clinical expression of AD (Snowdon et al., 1997). It is possible that identification of a receptor and blocking its interactions with Aβ may have therapeutic ramifications in the treatment of both HCHWA-D and the cerebrovascular aspects of AD.

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