Fibril Formation and Neurotoxicity by a Herpes Simplex Virus Glycoprotein B Fragment with Homology to the Alzheimer's Aβ Peptide[†]

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ABSTRACT: Despite significant progress in the elucidation of the genetic basis of early-onset familial Alzheimer's disease (AD), the etiology of sporadic cases remains elusive. Although certain genetic loci play a role in conferring susceptibility in some sporadic AD cases, it is likely that the etiology is multifactorial; hence, the majority of cases cannot be attributed to genetic factors alone, indicating that environmental factors may modulate the onset and/or progression of the disease. Head injury and infectious agents are environmental factors that have been periodically implicated, but no plausible mechanisms have been clearly identified. With regard to infectious agents, speculation has often centered on the neurotropic herpes viruses, with herpes simplex virus 1 (HSV1) considered a likely candidate. We report that an internal sequence of HSV1 glycoprotein B (gB) is homologous to the carboxyl-terminal region of the Aβ peptide that accumulates in diffuse and neuritic plaques in AD. Synthetic peptides were generated and the biophysical and biological properties of the viral peptide compared to those of Aβ. Here we show that this gB fragment forms β-pleated sheets, self-assembles into fibrils that are thioflavin-positive and ultrastructurally indistinguishable from Aβ, accelerates the formation of Aβ fibrils in vitro, and is toxic to primary cortical neurons at doses comparable to those of Aβ. These findings suggest a possible role for this infectious agent in the pathophysiology of sporadic cases of AD.

Genetic linkage analyses have identified several loci that underlie familial Alzheimer's disease (AD).[1] Mutations in the amyloid precursor protein (APP) gene and the presenilins (PS1 and PS2) have been linked to autosomal dominant cases of early-onset AD (*1*). The overwhelming majority of AD cases, however, originate sporadically, and their causes are not known. Certain genetic loci, such as the apolipoprotein E (apoE) locus, modulate an individual's susceptibility to AD and the age of onset (*2*), but are insufficient to cause AD, indicating that other factors, including unidentified genetic loci and perhaps environmental factors, may also be involved. Several potential environmental risk factors have occasionally been considered to contribute to AD neurodegeneration, including heavy metals such as zinc (*3*), traumatic events such as head injury (*4*, *5*), and conventional and unconventional infectious agents (*6*).

Viral agents have periodically been implicated in the etiology of sporadic AD (*7*−*9*). Although AD generally is not regarded as a transmissible disease, some viruses underlie certain neurological disorders that are not considered transmissible in conventional terms (e.g., measles virus in subacute sclerosing panencephalitis) (*8*). Virally mediated events could lead to neuronal stress and contribute to plaque or neurofibrillary tangle formation. Notably, it has recently been reported that the prevalence of Aβ argyrophilic plaques is significantly higher in the brain of AIDS patients compared to healthy control subjects (*10*), which is likely due to the AIDS virus triggering an inflammatory response. Therefore, it is not unprecedented that a virus could play a role in plaque formation, either through mediating inflammatory responses or perhaps through more direct means such as contributing proteins that serve as "seeds" for Aβ plaque formation.

In view of the large number of sporadic AD cases, if a virus did act as a cofactor for AD, it is likely to be a ubiquitous agent. HSV1 satisfies this criterion and has previously been considered a plausible candidate due to the large number of infected individuals in the population, estimated at 80−90% seropositivity (*7*). Other circumstantial evidence also implicates HSV1 (reviewed in ref *11*), including well-described anatomical routes of viral dissemination to the temporal lobe and limbic system, major sites of AD

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[1] Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; apoE, apolipoprotein E; APP, amyloid precursor protein; CD, circular dichroism; gB, glycoprotein B; Gly, glycine; HSV1, herpes simplex virus type 1.

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pathology, from the trigeminal ganglion or olfactory bulb (*7*, *12*). In addition, the regions of the brain that are most severely affected in herpes encephalitis are comparable to those that are predominantly affected in AD, with memory loss sometimes documented in individuals suffering from HSV1 encephalitis (*13*−*15*). Perhaps the strongest supporting evidence comes from several independent laboratories which have utilized PCR-based approaches to detect HSV1 DNA in AD-relevant regions of the aged human brain (*9*, *16*−*18*).

In this study, we report that glycoprotein B (gB) of HSV-1 is homologous to the Aβ peptide. The greatest degree of sequence similarity occurs near the carboxyl-terminal portion of Aβ, the region to which properties associated with neurotoxicity and fibril formation have been localized. The biophysical properties of the two peptides are similar on the basis of circular dichroism (CD) measurements, hydrophobicity profiles, fibril formation, and in vitro toxicity assays. In addition, the gB peptide can nucleate assembly of Aβ in vitro. These data, in combination with recent findings demonstrating the presence of HSV-1 DNA in brain, support the hypothesis that neurotropic herpes viruses may serve as a risk factor, which along with other genetic or environmental factors may affect the onset or progression of this insidious neurodegenerative disorder.

MATERIALS AND METHODS

Peptide Synthesis. Peptides were synthesized at the Peptide Facility of the University of California, Irvine. Peptides corresponding to amino acids 22−42 of HSV1 gB and Aβ were synthesized by solid-phase F-moc amino acid substitution on a model 9050 peptide synthesizer (Millipore) and then purified by reverse-phase HPLC as previously described (*19*). A scrambled version of the gB peptide (GVGAD-VRGSVMVGKGVALVIG) was also synthesized and studied. In addition, freshly prepared full-length $A\beta_{1-42}$ peptide was synthesized and used in the seeding experiments. Protein sequence alignments and analysis were conducted with the Protean program in Lasergene (DNASTAR, Madison, WI).

Analysis of Fibril Formation. For visual assessment of peptide aggregation, aliquots of each peptide were examined under phase-contrast microscopy for the presence of fibrillar and/or sheetlike precipitates. After inspection, peptide solutions were air-dried on gelatin-coated microscope slides, then stained with 1% (w/v) thioflavin S solution, rinsed with 50% ethanol, and examined microscopically under fluorescent illumination. For ultrastructural analysis, 25 μM samples of Aβ and gB peptides in 20 mM MOPS (pH 7.4) were adsorbed onto 200 mesh Formvar grids and stained with 2% uranyl acetate prior to viewing with a Zeiss 10CR transmission electron microscope at 80 kV transmission (*20*).

CD spectra were taken on a Jasco spectropolarimeter (model J-720) 24 h after peptide dissolution in Tris-buffered saline (pH 7.4) and are reported in units of mean residue ellipticity (degrees cm^2 $dmol^{-1}$). Data were taken with a 0.2 nm step size and a 1 s average time and were averaged over four scans.

Nucleation of Aβ Assembly. The level of the Aβ peptide assembly state was measured by the thioflavin T assay (Sigma, St. Louis, MO) in which the fluorescence intensity reflects the degree of aggregation (*21*). The gB peptide was preassembled by incubating in PBS (pH 7.2) at 25 °C for more than 3 days; the assembly state was verified by turbidity and EM analysis. Aliquots of preassembled gB peptide were added to freshly prepared Aβ, present at final concentrations of 80 and 100 μM in PBS. Solutions were allowed to incubate over 72 h at 25 °C, during which aliquots were removed at various times and assayed for peptide aggregation by the thioflavin T method and turbidity measurements (*22*) (data not shown). Aliquots of the mixture (10 μL) were added to 990 μL of 10 μM thioflavin T in 50 mM sodium phosphate buffer (pH 6.0). Solutions were mixed by vortexing, and the fluorescence intensity was immediately read at $E_x = 450$ nm and $E_m = 482$ nm using a SPEX Fluorolog F112A spectrofluorometer. The fluorescence intensity for 10 μM thioflavin T in the absence of peptide was determined and subtracted from those of peptide-containing samples as was the background fluorescence for 20 μM gB.

Analysis of the gB Peptide on Primary Cortical Neurons. To study the neurotoxic properties of the gB peptide fragment, primary cortical neurons prepared from rat brain as described previously (*23*) were grown in culture and exposed to the synthetic Aβ, gB, or scrambled peptides at various concentrations. Cell death was measured by two independent investigators by direct visual observations as previously described (*24*). To investigate the effect gB peptide treatment had on cellular APP levels, rat primary cortical neurons were treated with gB peptide at 12 μM and protein extracts prepared at various time points. Extracts were prepared in RIPA buffer containing leupeptin (1 μg/mL) and PMSF (50 μg/mL) at 4 °C. The supernatant was collected, and equal amounts of protein (30 μg) were fractionated on a 10% acrylamide/SDS gel, and transferred to PDVF membrane. The membrane was blocked in 5% nonfat dry milk in PBS and incubated overnight with antibody 22C11 to APP (Roche Molecular Biochemicals, Indianapolis, IN) as described previously (*24*, *25*). The blot was incubated with secondary antibody and developed using the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL).

RESULTS

Biophysical Analysis of the gB Fragment. We found that the gB protein of HSV1 contains a transmembrane sequence that is significantly homologous to the carboxyl-terminal region of Aβ (Figure 1a). It is precisely within this region of Aβ where the putative toxic (*26*) and the nucleation and assembly (*27*, *28*) domains reside. In accord with the hypothetical model for amyloid fibril formation proposed by Jarrett and Lansbury, the homologous regions of both peptides are highly hydrophobic and rich in β-branched amino acids, and contain two motifs with glycine as the fourth residue (presumably allowing greater conformational flexibility) (*22*). Moreover, $A\beta_{34-42}$ is 67% identical with the gB sequence, which is significant as this region is expected to initiate strong intermolecular interactions that result in self-association and formation of an antiparallel cross-β fibrillar core (*27*). The formation of a cis amide bond (Gly37−Gly38) surrounded by hydrophobic amino acids in Aβ also appears to be important for fibril formation (*29*), and it is notable that gB contains the Gly−Gly sequence at a comparable position. The gB peptide also has vicinal phenylalanine residues which are part of a hydrophobic core around amino acids 17−20 that have been shown to be

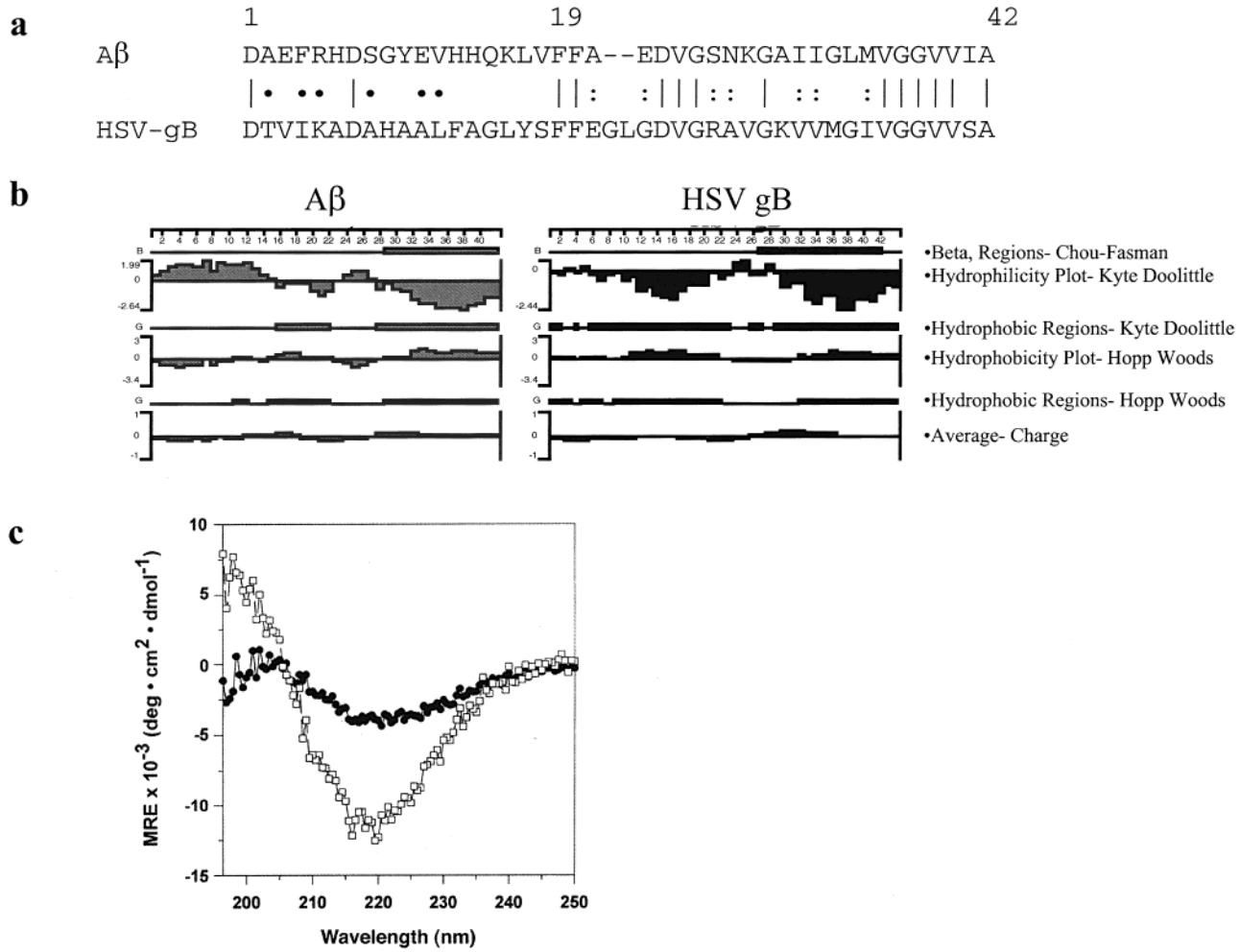

FIGURE 1: Sequence homology between the Alzheimer's Aβ and the HSV1 gB proteins. (a) The amino acid (single-letter code) sequences of the Aβ and gB peptides are shown in alignment, with vertical and broken lines indicating identical and related amino acids, respectively, as determined by Protean; conservation in codons is indicated with a single dot. Note that the region in which the greatest degree of homology exists (i.e., residues 22−42) is clustered in the region of Aβ through which it exerts its neurotoxicity and self-assembles into fibrils. (b) The gB and Aβ peptides are chemically similar, particularly in the region of residues 22−42, on the basis of predicted β regions (using the Chou−Fasman method), hydrophobicity−hydrophilicity profiles (using both Kyte−Doolittle and Hopp−Woods methods), and average charge distribution. (c) Circular dichroism analysis of the Aβ and gB synthetic peptides reveals substantial β-sheet conformation to the gB peptide. Each peptide was measured at 50 μM in aqueous buffer: (●) Aβ and (□) gB.

critical for Aβ assembly (*30*). The distribution of charged residues is similar for the two proteins, which is likely to be a critical factor for β-sheet formation (*31*, *32*) and for the fibrils to display a topochemical landscape necessary for biological activity. In addition, the gB protein shares additional sequence elements with Aβ that indicate that it may be comparably processed to form amyloidogenic fragments. At the amino terminus, both peptides begin with an aspartyl residue and end at the carboxyl terminus with an alanyl residue, which may represent putative β- and γ-secretase sites, respectively.

To determine whether the Aβ-like region of gB exhibits similar physical and biological properties, we synthesized a fragment of the gB sequence with the greatest degree of homology to Aβ. In this study, we paralleled the approach used to extensively characterize other amyloidogenic proteins such as Aβ peptide (e.g., Aβ_{25-35}) (*26*−*28*), prion protein (*33*), islet amylin (*34*, *35*), and bacterial protein OsmB (*29*). A synthetic gB peptide corresponding to amino acid residues 22−44 (gB$_{22-44}$) was purified by HPLC (*19*) and used for all subsequent experiments (see Figure 1). (Production and analysis of a synthetic gB peptide comparable to Aβ_{1-42} were initiated and revealed that this fragment readily aggregates to form thioflavin S-positive structures under physiological conditions; attempts, however, to purify this gB$_{1-42}$ peptide by HPLC failed due to its inherent insolubility characteristics.)

A distinguishing biophysical feature of amyloidogenic proteins such as Aβ is their ability to self-assemble in vitro into fibrils that are rich in β-sheet content. Circular dichroism (CD) measurements indicated that the gB fragment exhibited substantially more β-sheet structure than Aβ (Figure 1c). Since the self-assembly properties of the Aβ peptide underlie its toxic properties (*36*), we next investigated whether the HSV1 gB protein fragment could likewise self-assemble into fibrils. Notably, in both water and culture medium, the gB peptide rapidly formed fibrils that were similar to those observed with Aβ by light and electron microscopy (Figure 2a,b,e,f). The gB fragment was also capable of producing thioflavin S-positive structures comparable to those formed by the Aβ peptide (Figure 2c,d). Furthermore, electron microscopic analysis revealed that the gB peptide assembled

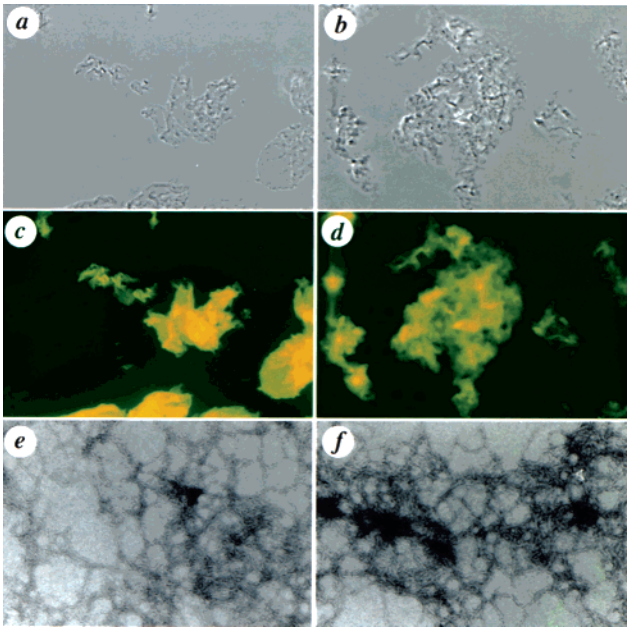

FIGURE 2: Light and electron micrographs of the assemblies formed by the Aβ and gB peptides. Aβ and gB peptides are shown on the left and right side, respectively: (a and b) light microscopy-phase contrast view, (c and d) thioflavin S staining of the peptides, and (e and f) electron micrographs of negatively stained peptide assemblies. Original magnifications were as follows: a, b, e, and f, 20×; c and d, 40000×. The image has been reduced 78%.

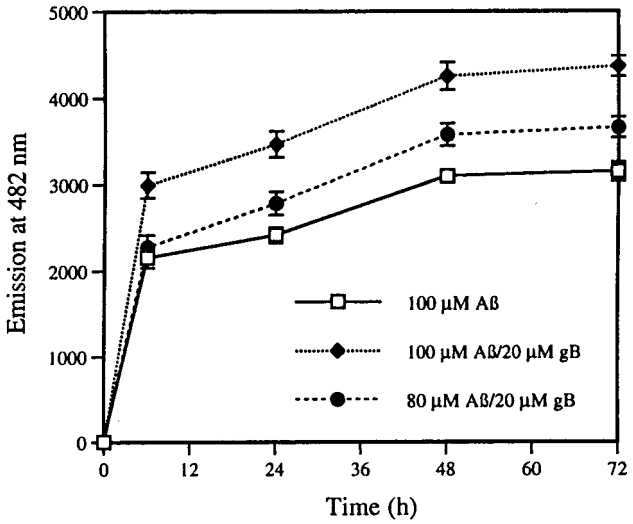

FIGURE 3: gB peptide accelerates the assembly of Aβ fibrils in vitro. Preassembled gB (20 μM final concentration) was added to freshly prepared solutions of 80 μM Aβ (●) or 100 μM Aβ (◆), and the mixtures were incubated for 72 h in PBS. The kinetics of 100 μM Aβ without any added gB were also determined (□). Aliquots of each sample were monitored for peptide aggregation by the thioflavin T assay at 6, 24, 48, and 72 h. Each point represents the mean ± standard deviation for triplicate determinations. The background fluorescence exhibited by the 20 μM gB fragment has been subtracted from all measurements.

into long uniform fibrils comparable to Aβ (Figure 2e,f). Therefore, this fragment of the gB protein exhibits the biophysical properties of a self-aggregating protein.

Previous studies have shown that preassembled Aβ can nucleate the aggregation of monomeric preparations of Aβ (*28*). Accordingly, we investigated whether the gB peptide could accelerate the formation of Aβ in vitro. We incubated preassembled gB fragments with freshly prepared monomeric Aβ and followed the kinetics of assembly using the thioflavin T assay (*21*). The gB peptide increased the rate and the final level of assembly of Aβ compared to samples that contained Aβ alone (Figure 3). Likewise, in a complementary experiment, preassembled Aβ peptide was also found to nucleate assembly of gB (data not shown). Since gB can seed and accelerate Aβ fibril formation, these data point to a potential mechanism by which reactivation of the herpes virus may play a role in AD neurodegeneration.

Neurotoxic Properties of gB. Perhaps the most important property associated with the Aβ peptide is its ability to initiate various signal transduction cascades which in neurons can lead to cell death both in vitro and in vivo (*26*, *37*−*39*). Many studies have indicated that the toxicity mediated by Aβ is dependent on the conformational state of the peptide (i.e., fibrillar vs soluble), with fibrillar Aβ exhibiting greater neurotoxicity (*36*, *40*, *41*). To determine if the HSV1 gB fragment was neurotoxic, neuronal cells were harvested from the brains of neonatal rats, grown in culture, and treated with gB peptide at concentrations previously shown to produce marked toxicity with Aβ. Whereas untreated cells appear healthy and extend processes (Figure 4a), after gB treatment, the fibrillar assemblies of the gB peptide were clearly visible on the neuritic processes and soma of the neurons, and were associated with profound changes in neuronal morphology which were readily apparent by 12 h (Figure 4b). These changes included retraction of neurites, neuritic dystrophy, and reduction in somal diameter (Figure 4c), similar to those observed following treatment with Aβ. By 24 h, extensive cell death had occurred in the neuronal cultures. Dose−response curves were constructed for the gB and Aβ synthetic peptides, and a comparison of the relative neurotoxicities of the two peptides is shown in Figure 4d. Moreover, the scrambled gB peptide exhibited markedly less neurotoxicity than the authentic sequence (data not shown).

In three separate experiments, the gB peptide was found to be more neurotoxic on a molar basis than Aβ. These results demonstrate that the gB peptide self-assembles, that the assembled peptide is toxic, and that the toxicity associated with the assembled form is extremely potent. Neurons appear to be unusually sensitive to the presence of certain self-assembled peptides, which may contribute to common pathological processes.

Exposure of cortical neurons to Aβ fibrils results in a rapid and progressive increase in the levels of the different isoforms of amyloid precursor protein (APP) as well as a robust increase in the level of APP processing fragments (*24*). We exposed cortical neurons to the synthetic gB peptide to determine whether it also triggered an increase in the total cellular APP load. Analysis of protein extracts from gB-treated cortical neurons showed a pattern strikingly similar to Aβ-induced changes, with numerous APP immunopositive bands detected by Western blotting (Figure 5). This suggests distinct APP processing events and not random proteolysis in response to gB_{22-44}. As a result, these findings suggest that gB may stimulate amyloidogenic processes in neurons and other cell types (*24*, *42*).

DISCUSSION

On the basis of the observations described above, it is clear that the gB peptide is amyloidogenic in vitro and exhibits

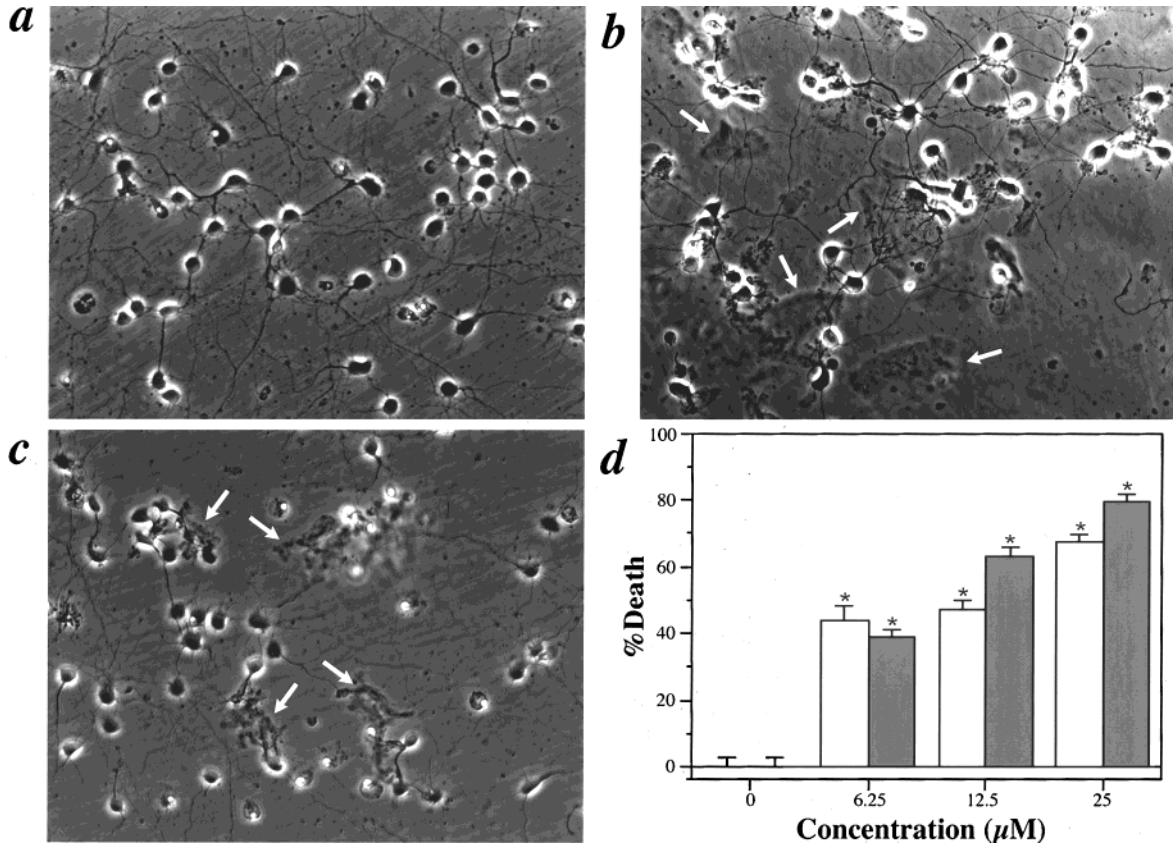

FIGURE 4: gB peptide causes degenerative changes in cultured neurons. Treatment of short-term cortical cultures with aggregated gB peptide (6 μM) results in distinct morphological changes within 24 h, comparable to changes observed following treatment with aggregated Aβ (6 μM). (a) Untreated control cells appear healthy with prominent neuritic processes. (b) Neurons treated with Aβ. (c) After gB treatment, most cells appear to be degenerative (cellular collapse); many large gB assemblies are also visible. (d) Dose−response curve comparing the toxic effect of various concentrations of the gB (gray) and Aβ (white) peptides following exposure to neurons. The extent of neuronal cell death was determined after 24 h by morphological cell counts. Bars represent mean percentage cell loss (±the standard error of the mean), and statistical analyses were performed using one-way ANOVA and the Scheffe *F*-test for post hoc between-group comparisons. Asterisks denote $P < 0.0001$. Arrows in b and c point to fibrillar peptide assemblies associated with neuritic processes.

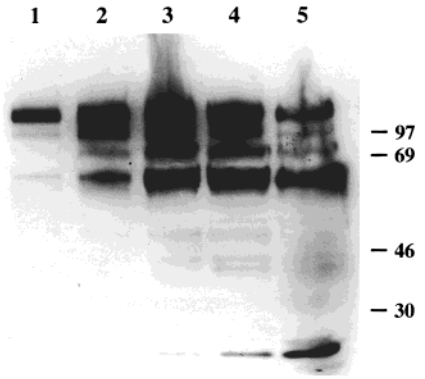

FIGURE 5: Exposure of gB peptide to neurons causes a time-dependent increase in the level of accumulation of APP and its breakdown products. Protein extracts were analyzed by Western blotting for APP using antibody 22C11. Lane 1 contained extract prepared from cells without any peptide treatment; lanes 2−5 contained extracts treated with the gB peptide for 3, 6, 12, and 24 h. Molecular mass markers are indicated in kilodaltons.

biological properties similar to those of Aβ. It is possible that either direct infection or, more likely, reactivation of the latent herpes virus may play a role in some cases of AD. Periodic reactivation of HSV can result in production of the infectious virus from latently infected neurons with or without the onset of clinical symptoms or herpes lesion formation (*43*). It has previously been demonstrated that herpes viruses can be reactivated in "stressed" neurons (*44*, *45*), and a similar situation could exist in the AD brain. Further, the biophysical properties of the gB protein render it a likely candidate for inducing neuronal degeneration. For instance, the intracellular processing of gB in neurons, as a result of viral reactivation, may lead to the generation of amyloidogenic fragments with the potential to nucleate the assembly of the constitutively produced Aβ. This is analogous to the situation in which the assembly of the kinetically "slow nucleating" Aβ_{1-40} can be efficiently propagated by the kinetically "fast nucleating" Aβ_{1-42} (*28*). In this regard, previous studies have demonstrated that aberrant processing of gB leads to its retention in the endoplasmic reticulum (*46*), where it may potentially serve as a seed for Aβ nucleation. This is particularly relevant since we demonstrate that the gB peptide can accelerate the assembly of Aβ fibrils in vitro. In addition, gB-induced neurodegeneration may stimulate a recurring cascade that could further facilitate Aβ production. The APP gene promoter contains stress response elements that have been shown to induce increased expression levels following activation (*47*).

Although the sequence similarity between gB and Aβ may be fortuitous, it is relevant to note that an amino-terminal region of the gB protein has been reported to resemble the amino acid sequence in apolipoprotein E (apoE) that is involved in the binding of apoE to LDL receptors (*48*).

Notably, the HSV1 gB protein has also been shown to bind to human serum lipoproteins, including apoE (*49*). The HSV1 gB protein, therefore, contains two separate domains that resemble two different proteins that have been strongly correlated with the pathophysiology of AD. In view of the results presented here, the specter that herpes viruses may act as a cofactor or risk factor in AD warrants further consideration.

The linkage of a viral agent to the pathogenesis of AD is likely to be difficult due to the inherent uncertainty in establishing the time point when an infectious agent may be involved. A virus may plausibly act during the early stages of the disease process yet no longer be required or evident during terminal stages; this is of course analogous to the situation that occurs with human papilloma viruses and the development of cervical carcinoma in which papilloma viruses clearly cause the development of this neoplasm even though no virus is detectable at the terminal stages of disease (*50*). Most relevantly, HSV1 DNA is commonly found in the brains of elderly individuals in the same regions exhibiting pathology in AD (*51*, *52*). It is important to point out that the experiments conducted here utilized a synthetic fragment of the gB protein, and it remains to be determined whether the holoprotein is proteolytically processed in a manner comparable to that of APP. Notably, the gB fragment of interest, like Aβ, contains an aspartyl residue at the amino terminus and an alanyl residue at the carboxyl terminus that may represent putative β- and γ-secretase sites, respectively. Thus, it is plausible that the gB holoprotein undergoes proteolytic processing like APP to produce a peptide fragment that either is directly neurotoxic or serves as a "seed" to nucleate assembly of Aβ in the brain, exacerbating the neurodegenerative state.

The evidence presented here provides a potential mechanism by which HSV1 contributes to brain pathology. Itzhaki and her colleagues have reported that the risk of developing AD appears to be much greater in those individuals who both are infected with HSV1 and possess an apoEe4 allele than in those individuals with only one of these risk factors (*9*, *53*); other investigators have been able to reproduce these findings to some extent (*17*, *18*, *54*). Moreover, interactions of neurotropic viruses with E4 may not be unique to HSV1, as HIV-infected patients harboring the E4 allele have an increased rate of dementia and peripheral neuropathy (*55*). Although further studies will be required, our findings indicate that basic investigations into the possible presence, processing, and assembly of gB from neurotropic herpes viruses in particular populations of individuals may help elucidate the onset and course of this neurodegenerative disease.

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