Reactive microglia associated with the β-amyloid plaques in Alzheimer’s disease (AD) brains initiate a sequence of inflammatory events integral to the disease process. We have observed that fibrillar β-amyloid peptides activate a tyrosine kinase-based signaling response in primary mouse microglia and the human monocytic cell line, THP-1, resulting in production of neurotoxic secretory products, proinflammatory cytokines, and reactive oxygen species. We report that most of the amyloid-induced tyrosine kinase activity was stimulated after activation of Src family members such as Lyn. However, transcription of the signaling response required for increased production of the cytokines TNFα and IL1-β was mediated by the nonreceptor tyrosine kinase, Syk. Additionally, β-amyloid stimulated an NFκB-dependent pathway in parallel that was required for cytokine production. Importantly, TNFα generated by the monocytes and microglia was responsible for the majority of the neurotoxic activity secreted by these cells after β-amyloid stimulation but must act in concert with other factors elaborated by microglia to elicit neuronal death. Moreover, we observed that the neuronal loss was apoptotic in nature and involved increased neuronal expression of inducible nitric oxide synthase and subsequent peroxynitrite production. Selective inhibitors of inducible nitric oxide synthase effectively protected cells from toxicity associated with the microglial and monocyctic secretory products. This study demonstrates a functional linkage between β-amyloid-dependent activation of microglia and several characteristic markers of neuronal death occurring in Alzheimer’s disease brains.

Key words: Alzheimer’s disease; β-amyloid; microglia; THP-1 monocytes; signal transduction; tyrosine kinase; Lyn; Syk; inflammation; neurotoxicity; apoptosis; nitric oxide; nitrotyrosine; peroxynitrite; TNFα; cytokines

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recent in vitro studies have demonstrated that Aβ fibril stimulation increases microglial/monocytic TNFα production (Klegeris et al., 1997; Galimberti et al., 1999; Combs et al., 2000; Yates et al., 2000). Importantly, increased levels of TNFα have been reported in brains and plasma of AD patients (Fillit et al., 1991; Brunnsgaard et al., 1999; Tarkowski et al., 1999).

We report that fibrillar Aβ stimulation of mouse microglia and THP-1 monocytes results in a Syk kinase and NFκB-dependent production of TNFα that is responsible for increased iNOS expression, peroxynitrite production, and subsequent apoptosis in primary mouse neuronal cultures. These data establish a functional linkage between Aβ-stimulated microglial proinflammatory changes and the specific characteristics of neuron loss that occur in AD brains.

MATERIALS AND METHODS

Materials. The anti-MAP2 antibody was purchased from Sigma (St. Louis, MO). Anti-ERK2 and anti-c-fos antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). The anti-phosphotyrosine antibody, 4G10, was from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-CREB (Ser133) antibody was from New England Biolabs (Beverly, MA). Anti-active p56Lck (RelA) antibody and picatannol were purchased from Boehringer Mannheim (Mannheim, Germany). Anti-phospho-IκBα antibody was from New England Biolabs. Anti-IκBα antibody and PP1 were purchased from Calbiochem (La Jolla, CA). Anti-active p38 and active-MAP kinase antibodies were from Promega (Madison, WI). Anti-nitrotyrosine antibody and NFκB SN-50 cell-permeable inhibitory peptide were both obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Anti-human TNFα/IL-1β antibodies, mouse recombinant TNFα, and anti-mouse TNFα antibody were all purchased from R&D Systems (Minneapolis, MN). The anti-iNOS antibody as well as the specific iNOS inhibitors, 1400W,2HC1 and AMT.HCl, and the neuronal nitric oxide synthase (nNOS) inhibitor Vinyl-l-NIO were purchased from Alexis Biochemicals (San Diego, CA). Affinity-purified horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). FITC-conjugated goat anti-rabbit antibody was from Jackson Laboratories (Bar Harbor, ME). Peptides corresponding to amino acids 25–35 and 1–40 of human Aβ were purchased from Bachem (Philadephia, PA). β-amyloid peptides were resuspended in sterile dH2O. Fibrillar Aβ 1–40 and 25–35 peptides were prepared by reconstitution of the lyophilized peptides in sterile distilled water, followed by incubation for 1 week at 37°C. Neurobasal media and B27 supplements were purchased from Life Technologies (Rockville, MD).

Tissue culture. THP-1 cells are a monocytic cell line derived from peripheral blood of a human with acute monocytic leukemia and were purchased from the American Type Culture Collection (Manassas, VA). All experiments using THP-1 cells used undifferentiated cells. THP-1 cells were grown in RPMI-1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% heat-inactivated fetal calf serum, 5 × 10^-5 M 2-mercaptoethanol, 5 mM HEPES, and 2 μg/ml gentamicin in 5% CO2. Microglial and neuronal cultures were derived from postnatal day 1–2 and embryonic day (E) 16 mouse brain (C57BL/6), respectively, as described previously (Combs et al., 1999, 2000). Neurons were grown in Neurobasal media (4.0 × 10^4 per 24-well tissue poly-l-lysine coated tissue culture plate) with B27 supplement for 5 d in vitro before use.

Cell stimulation. THP-1 cells and microglia were stimulated as described previously (Combs et al., 1999, 2000). Briefly, monocytes and microglia were removed from their respective media and transferred to serum-free RPMI for suspension stimulation or to Neurobasal media for adherent stimulation. Adherent stimulation was performed by plating the cells onto Aβ peptide bound to the surface of the dish (48 pmol/mm^2). Bound fibrillar peptides were prepared as described previously (McDonald et al., 1997). Briefly, tissue culture wells were coated with nitrocellulose, and peptides were added to the coated wells and allowed to dry, immobilizing the peptides. The use of immobilized peptides prevented their subsequent collection medium and transfer to the neuronal cultures, avoiding any confounds arising from the action of amyloid fibrils on neurons. THP-1 cells and microglia (2.0 × 10^4 cells) were added to wells containing the bound peptides in 48-well tissue culture dishes in 0.5 ml of Neurobasal media for 48 hr. The conditioned media from these cells was clarified by centrifugation and added to neuronal cultures for 72 hr with vehicle (DMSO) or selected reagents. To determine the involvement of TNFα for the neuronal death, conditioned media was incubated with anti-human or anti-mouse (5 μg/ml media) TNFα-neutralizing antibodies for 15 min, 25°C before addition to neurons for 72 hr. The participation of IL-1β was evaluated similarly using an anti-human IL-1β antibody. Additionally, recombinant mouse TNFα (100 ng/ml) was added to neurons directly or into conditioned media with and without anti-human TNFα neutralizing antibody for 72 hr. The selective NOS inhibitors, 1400W,2HC1 (nNOS, 5 and 10 μM), AMT.HCl (iNOS, 10 μM) and Vinyl-l-NIO (nNOS, 20 μM) were also added to neurons in the absence or presence of conditioned media. Neurons were fixed, stained, and counted after staining of the cultures using a mouse anti-MAP2 antibody. A counting grid was placed over the wells to count neuron and astrocyte numbers from eight identical fields for each condition. The average number of neurons and astrocytes (±SEM) was calculated for each condition. Each experiment was performed in duplicate and repeated three to four times.

Western blotting. Cells were lysed in 200 μl of ice-cold RIPA buffer [1% Triton, 0.1% SDS, 0.5% deoxycholate, 20 mM Tris, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM Na2VO3, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)], and insoluble material was removed by centrifugation (10,000 × g at 4°C for 20 min). Protein concentrations were quantitated by the method of Bradford (1976). Proteins were resolved by 7.5–9% SDS-PAGE, and the resulting Western blots were incubated with primary antibodies overnight at 4°C followed by incubation in horseradish peroxidase-conjugated secondary antibodies (1:3000, 1 hr, 25°C). Antibody binding was detected via enhanced chemiluminescence (Pierce, Rockford, IL). Blots were re-probed by stripping with 0.2N NaOH and shaking vigorously for 10 min at 37°C (Suck and Krupinska, 1996).

Immunoprecipitation. To perform immunoprecipitations, cells were lysed in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 0.2% PMSF. Cell lysates were incubated with immunoprecipitating antibody, anti-p65NLS (1 μg antibody, 1:3000, 1 hr, 25°C), and Protein A-Sepharose beads for 2 hr at 4°C. The immunoprecipitates were washed three times in lysis buffer and then resolved by 7.5% SDS-PAGE and Western-blotted as described.

TNFα reporter assays. Luciferase reporter constructs for the human TNFα gene were transfected into THP-1 cells using DEAE-dextran together with a β-galactosidase reporter construct to control for transfection efficiency as described previously (Combs et al., 2000). The cells were transfected, and 48 hr later they were stimulated for 0–8 hr in serum-free RPMI media in the presence or absence of drugs/SN-50 peptide and fibrillar Aβ25–35 (60 μM) or Aβ1–40 (60 μM). The cells were lysed, and luciferase activity was measured and normalized to β-galactosidase activity. All assays were performed in duplicate in three separate experiments.

Immunocytochemistry. For immunocytochemistry, cells were fixed for 4% paraformaldehyde for 30 min at 37°C. To ensure the numbers of surviving cells, neurons were stained with a mouse anti-MAP2 antibody (1:500). Immunoreactivity was visualized using 3,3’-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories, Burlingame, CA). Immunodetection of iNOS and nitrotyrosine was performed using anti-iNOS (1:2000) and anti-nitrotyrosine (1:1000) antibodies and visualized using FITC-conjugated goat anti-rabbit antibody (1:500).

TUNEL protocol. After 72 hr treatment in the absence or presence of conditioned media, neurons were fixed in 4% paraformaldehyde for 30 min at 37°C and then DNase-treated for 15 min. The terminal transferase reaction was then performed for 1 hr at 37°C to allow incorporation of biotinylated dUTP. Biotinylated dUTP was visualized using the Vector Elite ABC reagent kit according to manufacturer’s protocol using DAB as the chromagen.

DNA ladder. After 48 hr treatment in the absence or presence of conditioned media, neurons were lysed in 5 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 20 mM EDTA, 50ug/ml RNase A, 0.2 mM PMSF, rocked at 4°C for 30 min, and spun (10 min, 16,000 rpm, 4°C). Endonuclease-cleaved DNA was phenol/chloroform-extracted and ethanol-precipitated from the supernatants and resolved by 1.8% agarose gel electrophoresis. Gels were stained with ethidium bromide for photography.

Statistical analysis. All experiments were performed in duplicate or replicate a minimum of three to four times. Mean values (±SEM) for each experiment were determined, and values statistically different from controls were calculated using one-way ANOVA. The Tukey-Kramer multiple comparisons post-test was used to determine p values.
RESULTS

**Aβ stimulation of THP-1 cells leads to activation of proinflammatory transcription factors**

We have previously described tyrosine kinase-based signaling pathways activated by stimulation of microglia and THP-1 monocytes with full-length fibrillar Aβ1–40, Aβ1–42, or its active domain, Aβ25–35 (McDonald et al., 1997, 1998; Combs et al., 1999). We and others (Yates et al., 2000) have documented that primary microglia and the THP-1 monocytes respond similarly after exposure to all fibrillar amyloid peptides. Because of this consistent similarity in responsiveness, we initially performed experiments using the THP-1 cell line and Aβ25–25 fibrils and subsequently confirmed that these responses are also elicited by the full-length Aβ1–40 peptide and are observed in primary microglial cultures. Stimulation of the cells with Aβ fibrils resulted in increased cellular protein phosphotyrosine levels resulting from activation of several tyrosine kinases, including the Src family members Lyn, Syk, focal adhesion kinase, and PYK2 (Fig. 1) (McDonald et al., 1997; Combs et al., 1999). Tyrosine kinase activation lead to the activation of both the ERK MAP kinase and the p38 MAP kinase pathways (Fig. 1) (McDonald et al., 1998; Combs et al., 1999). We investigated the Aβ-dependent phosphorylation and activation of transcription factors involved in the upregulation of proinflammatory genes in these cells. Aβ stimulation resulted in the phosphorylation of CREB at the regulatory Ser133, reflecting activation of its transcriptional activity (Fig. 1) (McDonald et al., 1998). Stimulation with Aβ also resulted in increased expression of c-fos, indicating AP-1 trans-

activation, consistent with previous reports (Yates et al., 2000). Importantly, Aβ stimulation resulted in activation of the proinflammatory transcription factor, NFκB. Exposure of the cells to Aβ fibrils resulted in the phosphorylation of IκBα followed by its proteolytic degradation (Karin, 1999). In parallel, increased levels of the active form of the Rel A (p65) subunit of the NFκB dimer were detected.

**Regulation of transcription factor activation through Lyn- and Syk-linked signaling pathways**

Nonreceptor tyrosine kinases such as the Src family members Lyn and Syk kinase serve as membrane proximal signaling elements in the Aβ-dependent inflammatory response (McDonald et al., 1997; Combs et al., 1999). To determine whether transcription factor activation relied on these tyrosine kinase activities, we preincubated the THP-1 cells with the Src family-specific inhibitor PP1 (5 μM) as well as the Syk-selective inhibitor piceatannol (10 μM) for 30 min before stimulation with fibrillar Aβ1–40 (60 μM) for 60 min. Cell lysates were resolved by 7.5% SDS-PAGE and Western-blotted with selected antibodies. The antibodies used were 4G10 (anti-phosphotyrosine), anti-phospho-ERK, anti-phospho-p38, anti-phospho-IκBα, anti-p65NLS, anti-c-fos, anti-phospho-CREB, and anti-ERK2.

**Figure 1. β-Amyloid fibrils stimulate activation of NFκB and multiple MAP kinase pathways in THP-1 cells.** THP-1 cells were stimulated in serum-free RPMI with fibrillar Aβ25–35 (60 μM) for increasing times (0–60 min). Cell lysates were resolved by 7.5% SDS-PAGE and Western-blotted with selected antibodies. The p65 (RelA) subunit of NFκB was immunoprecipitated from the stimulated cell lysates and resolved by 7.5% SDS-PAGE and Western-blotted. ERK2 levels were evaluated in parallel as a protein loading control. The antibodies used were 4G10 (anti-phosphotyrosine), anti-phospho-ERK, anti-phospho-p38, anti-phospho-IκBα, anti-p65NLS, anti-c-fos, anti-phospho-CREB, and anti-ERK2.

**Figure 2. Regulation of transcription factor activation through Lyn- and Syk-linked signaling pathways.** THP-1 cells were treated in serum-free RPMI with vehicle (DMSO) or 5 μM PP1 (Src inhibitor) or 10 μM piceatannol (Syk inhibitor) for 30 min before stimulation with fibrillar Aβ1–40 (60 μM) for 60 min. Cell lysates were resolved by 7.5% SDS-PAGE and Western-blotted with selected antibodies. The antibodies used were 4G10 (anti-phosphotyrosine), anti-phospho-ERK, anti-phospho-p38, anti-phospho-IκBα, anti-c-fos, anti-phospho-CREB, and anti-ERK2.
or the Aβ-dependent CREB phosphorylation (Fig. 2). However, piceatannol preincubation of THP-1 cells was able to completely eliminate the Aβ-dependent increase in c-fos expression (Fig. 2). Interestingly, pretreatment with either drug had no effect on inhibiting activation of NFκB as determined by decrease in IκB protein levels (Fig. 2) (Chen et al., 1996). These data demonstrate that Lyn and Syk each mediate a subset of the specific downstream signaling events elicited by Aβ treatment of the cells.

**Aβ-dependent increase in TNFα expression is dependent on Syk and NFκB activity**

Aβ fibrils stimulate an increase in monocyte/microglial proinflammatory cytokine production in vitro (Klegeris et al., 1997; Lorton, 1997; Meda et al., 1999; Combs et al., 2000; Yates et al., 2000). Aβ fibrils stimulated increased promoter activity for TNFα in transiently transfected THP-1 cells using a luciferase reporter linked to the human promoter elements of the TNFα gene (Fig. 3A). We linked the Aβ-dependent increase in TNFα production to the proximal signaling events by incubating TNFα–luciferase reporter-transfected THP-1 cells with Aβ1–40 and Aβ25–35 fibrils for 5 hr in the presence or absence of piceatannol, PP1, or the NFκB inhibitory peptide, SN-50. TNFα promoter activity required activation of NFκB and Syk but not Src family kinases (Fig. 3C). As reported previously, Aβ-stimulated levels of mature IL-1β were very low in these cells (Lorton et al., 1996).

**Conditioned media from Aβ-stimulated monocytes induces neuronal apoptosis**

Microglia and monocytes secrete neurotoxic factors on stimulation with Aβ-fibrils (Combs et al., 1999, 2000). However, the nature of the neurotoxic factors has not been clearly defined (Banati et al., 1993; Giulian et al., 1995; Li et al., 1996; Klegeris et al., 1997; Combs et al., 1999, 2000). We have investigated the method of neuronal death that occurs after stimulation with conditioned media from Aβ-stimulated THP-1 cells. Several studies have documented the presence of markers of neuronal apoptosis in AD brains as evidenced by increased immunoreactivity for active caspases and endonuclease-cleaved DNA (Li et al., 1997; Selznick et al., 1999; Stadelmann et al., 1999). To verify that the method of microglial/microcytic-dependent death in our in vitro system was duplicating disease-related phenomena, we treated cortical neuron cultures for 48 hr using conditioned media from THP-1 cells stimulated for 48 hr with Aβ1–40 fibrils. To assess whether neurons were dying apoptotically, cultures were fixed and TdT-mediated dUTP nick end labeling (TUNEL) was performed to visualize the endonuclease-cleaved DNA. Conditioned media treatment resulted in a clear increase in numbers of TUNEL-labeled neuronal nuclei compared with control cultures (Fig. 4A,B), and the DNA obtained from the neuronal cultures displayed a characteristic DNA ladder reflecting DNA fragmentation (Fig. 4C).
Neuronal apoptosis produced by stimulation with conditioned media from Aβ-stimulated monocytes/microglia occurs in a TNFα/iNOS-dependent fashion

Activation of microglia or monocytes results in increased secretion of a number of proinflammatory products. We tested whether TNFα secreted by the monocytes/microglia was responsible for the apoptosis that we had observed. TNFα has a well described ability to synergize with other stimuli, resulting in apoptosis of a number of cell types (Fiers, 1991; Natoli et al., 1998). Moreover, TNFα-dependent neuronal apoptosis has been linked to the increased expression and activity of iNOS (Ogura et al., 1997; Heneka et al., 1998; Chung et al., 1999). Importantly, it has been reported that levels of TNFα and neuronal iNOS immunoreactivity are increased in the AD brain (Fillit et al., 1991; Vodovotz et al., 1996; Bruunsgaard et al., 1999; Tarkowski et al., 1999). We investigated whether conditioned media from Aβ-stimulated monocytes and microglia induced neuronal apoptosis in a manner requiring TNFα and iNOS activity. To determine whether TNFα was required for neuronal apoptosis, conditioned media from Aβ1-40 and Aβ25-35 stimulated microglia and monocytes was preincubated with neutralizing antibodies to mouse or human TNFα, respectively, before addition to neuronal cultures. Antibody preincubations completely attenuated the neuron loss caused by the conditioned media (Fig. 5). We determined that neutralizing antibodies for IL-1β did not improve neuron survival in the presence of conditioned media (Fig. 5). Importantly, addition of mouse TNFα to THP-1 cell-conditioned media containing anti-human-specific TNFα neutralizing antibody restored the ability of the media to elicit neuron death (Fig. 5). These data demonstrate that TNFα is responsible for much of the neuronal death observed in these cultures. However, direct administration of recombinant mouse TNFα to neurons had no effect on neuron survival (Fig. 5). These findings demonstrate that additional factors present in the conditioned media act in concert with TNFα to elicit apoptosis, consistent with the well described requirement of TNFα to act synergistically with other agents to signal an apoptotic response (Venters et al., 1999, 2000).

We determined the involvement of neuronal iNOS activity in the conditioned media-dependent neuron death by adding iNOS-selective inhibitors to the media at the time of addition to the neuron cultures (Tracey et al., 1995; Garvey et al., 1997). The iNOS-selective inhibitors, AMT.HCl and 1400W.2HCl, blocked neuron death associated with treatment using either microglial or monocytic Aβ1-40 and Aβ25-35-stimulated conditioned media (Fig. 6A,B). To determine whether nNOS activity also contributed to the neuronal death caused by conditioned media, the nNOS-specific inhibitor, Vinyl-l- NIO, was added to neurons at the time of addition of conditioned media (Babu and Griffith, 1998). In contrast to the results obtained with iNOS-selective inhibitors, Vinyl-l-NIO had no ability to ameliorate conditioned media-dependent death.

TNFα stimulates the increased expression of iNOS in neuronal cell types (Ogura et al., 1997; Heneka et al., 1998; Chung et al., 1999). The requirement of iNOS activity for neuronal apoptosis in our system correlated well with increased neuronal iNOS expression in cultures treated with conditioned media. Neurons treated with Aβ1-40-stimulated THP-1-conditioned media displayed increased iNOS immunoreactivity (Fig. 7A,B). Addition of the anti-human TNFα antibody to the conditioned media prevented the increase in iNOS immunoreactivity after 72 hr of stimulation (Fig. 7C). Similarly, addition of mouse TNFα alone was able to induce an increase in iNOS immunoreactivity (Fig.
The TNFα-dependent increase in conditioned media-stimulated iNOS expression was verified by Western blot analysis of lysates from the treated neurons (Fig. 7E).

**Conditioned media-treated neurons display increased nitrotyrosine levels indicative of peroxynitrite production**

One of the consequences of increased inducible nitric oxide synthase expression is the elevation of intracellular nitric oxide levels. Increased concentrations of nitric oxide favor the rapid reaction with superoxide anion to produce the strongly oxidizing agent peroxynitrite (Ischiropoulos et al., 1992a,b). In addition to the well known ability of peroxynitrite to mediate oxidative damage to sulfhydryl groups and lipid peroxidation, it also reacts with Cu, Zn, Mn, and Fe superoxide dismutase, enabling it to nitrate protein tyrosine residues (Beckman et al., 1992; Ischiropoulos et al., 1992a,b). Although the consequences of protein tyrosine nitration are not entirely clear, protein nitrotyrosine levels can be used as an indirect measure of peroxynitrite production (Beckman et al., 1992; Ischiropoulos et al., 1992a,b). Importantly, neurons in AD brains display increased nitrotyrosine immunoreactivity, indicative of increased peroxynitrite production (Good et al., 1996; Vodovotz et al., 1996). We asked whether our in vitro paradigm leading to increased neuronal iNOS expression would replicate AD-associated phenomenon and produce increased nitrotyrosine immunoreactivity. We found that neurons treated with Aβ-stimulated conditioned media from microglia and THP-1 cells displayed increased nitrotyrosine immunoreactivity relative to untreated control cultures (Fig. 8A–D). The basal level of nitrotyrosine observed in these experiments is a consequence of elevated iNOS levels arising from in vitro culture conditions. Interestingly, addition of the iNOS-selective inhibitor 1400W.2HCl to conditioned media-treated cultures was able to completely eliminate the nitrotyrosine immunoreactivity (Fig. 8E,F). Immunoprecipitation of nitrotyrosine-containing proteins from conditioned media-treated and control cultures revealed a selective increase in nitration of several high molecular weight species (Fig. 8G).

**DISCUSSION**

Our previous reports have characterized tyrosine kinase-based inflammatory signaling pathways activated in microglia and THP-1 monocytes with Aβ fibrils, resulting in production of proinflammatory cytokines, secretion of superoxide anions, and 7D). The TNFα-dependent increase in conditioned media-stimulated iNOS expression was verified by Western blot analysis of lysates from the treated neurons (Fig. 7E).

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generation of neurotoxic products (McDonald et al., 1997, 1998; Combs et al., 1999, 2000). Numerous other reports have documented similar findings from microglial lineage cells after Aβ stimulation (Del Bo et al., 1995; Giulian et al., 1995; Lorton, 1996, 1997; Klegeris et al., 1997; Meda et al., 1999; Yates et al., 2000). We also demonstrated that treatment of monocytes with specific enzymatic inhibitors that target enzymes activated in the Aβ response were sufficient to prevent production of neurotoxins (Combs et al., 1999). Treatment of microglia and monocytes with activating ligands for the nuclear receptor PPARγ were also capable of preventing the Aβ-dependent production of neurotoxic factors (Combs et al., 2000). This protection is likely afforded through the ability of PPARγ to prevent NFκB and AP-1-dependent proinflammatory transcriptional events (Lemberger et al., 1996; Ricote et al., 1998). The present report verifies that the Aβ-stimulation of monocytes results in the activation of transcription factors involved in proinflammatory gene expression, such as NFκB and AP-1, and links this response to the previous activation of tyrosine kinase-dependent signaling events. Moreover, we have identified TNFα as the principal neurotoxic agent resulting from the proinflammatory transcriptional changes. Finally, we demonstrate that the mechanism of TNFα-mediated neuronal death is iNOS-dependent apoptosis. These data provide a mechanistic explanation of how fibrillar Aβ stimulation of microglia results in production of proinflammatory products and ultimately the oxidative damage-associated neuronal apoptosis observed in AD brains (Fig. 9).

Increased expression of proinflammatory cytokines by microglial lineage cells is a well described phenomenon that occurs in response to numerous activating stimuli. We as well as others (Klegeris et al., 1997; Lorton, 1997; Combs et al., 2000; Yates et al., 2000) have reported the increased expression of TNFα and IL-1β in microglial lineage cells in response to Aβ fibril stimulation. Interestingly, Aβ-dependent production of cytokines does not require the activity of Src-related kinases, although most...
stimulated tyrosine kinase activity is linked to Src family activation (Combs et al., 1999). Moreover, the Aβ-dependent increase in p38 and ERK MAP kinase activities as well as the subsequent phosphorylation of CREB and increase in c-fos expression required Src family member activation. In contrast, the Syk-selective inhibitor piceatannol was not effective at preventing the Aβ-dependent increase in protein phosphotyrosine levels, MAP kinase activities, or CREB phosphorylation, but dramatically prevented increased c-fos expression. It is critical to note that we have used a piceatannol concentration (10 μM) lower than that reported in our previous report (Combs et al., 1999). The lower drug concentration was required to maintain specificity of enzyme inhibition. Most importantly, Syk, but not Src, inhibition resulted in inhibition of the Aβ-dependent increase in production of IL-1β and TNFα promoter activity. This is in agreement with our previous observation that Src kinase inhibitors were not effective at preventing the Aβ-dependent respiratory burst in these cells (Combs et al., 1999). These data suggest a bifurcation of Aβ-mediated signaling in microglia in which increased cytokine production involves a mechanistically distinct pathway from other parallel signaling cascades.

Importantly, inhibition of active NFκB nuclear translocation using the SN-50 peptide prevented the Aβ-stimulated increase of TNFα promoter activity. Interestingly, neither Src family nor Syk kinase inhibition prevented IκB degradation. These data suggest that the Aβ-mediated activation of the NFκB pathway relies on yet another class of membrane proximal signaling elements and demonstrates the functional redundancies used by these cells for regulating proinflammatory gene expression.

A major question unanswered by our previous studies was the identity of the neurotoxic factor(s) in our culture system as well as the characterization of the neuronal death. We now identify TNFα as a critical element required for iNOS-dependent apoptosis. TNFα actions observed here are transduced through binding to TNF receptor I because human TNFα binds selectively to TNF receptor I and not TNF receptor II (Fiers, 1991). There is an extensive and conflicting literature concerning the ability of TNFα to induce both pro-apoptotic (Sipe et al., 1996; Ogura et al., 1997; Heneka et al., 1998; Chung et al., 1999; Sortino et al., 1999; Venters et al., 1999, 2000) and anti-apoptotic (Cheng et al., 1997; Heneka et al., 1998; Chung et al., 1999; Sortino et al., 1999) responses in neuronal cells. Importantly, the pro-apoptotic action of TNFα on primary neurons typically requires its specific presentation to neurons as part of an inflammatory milieu such as that derived from glial cells (Gelbard et al., 1993; Chao and Hu, 1994; Viviani et al., 1998; Downen et al., 1999; Venters et al., 1999). Similarly, the differential actions of TNFα on cellular survival has been rigorously examined in a number of cell types (Rath and Aggarwal, 1999; Sethi and Hotamisligil, 1999; Wallach et al., 1999; Smythe and Johnstone, 2000). It is now clear that divergent signaling pathways downstream of TNF receptor I lead to either cell survival or death (Nalotli et al., 1998). In many cell types, the default TNFα signaling pathway is anti-apoptotic unless additional stimuli such as other cytokines or RNA/protein synthesis inhibitors are also applied (Fiers, 1991; Nalotli et al., 1998; Xu et al., 1998; Jones et al., 2000). We have arrived at similar conclusions in interpreting the outcome of our experiments demonstrating that the apoptosis-inducing action of the conditioned media on neurons required both TNFα and an additional factor(s) that acts synergistically to promote cell death. TNFα alone was without effect on neuron survival. Indeed, recent data favoring this hypothesis demonstrated that the TNFα signaling pathway cross-talks with pathways used by the insulin-like growth factor-1 (IGF-1) receptor to result in neuronal death. These findings dramatically illustrate that TNFα can act not only as a neurotrophic factor but also as a “silencer of survival signaling” (Loddick and Rothwell, 1999; Venters et al., 1999, 2000).

The TNFα-dependent neuronal apoptosis observed in our experiments revealed a dependence on iNOS activity. This requirement for iNOS activity correlated well with increased iNOS expression after treatment of cortical neurons with Aβ-stimulated conditioned media. More importantly, the increase in iNOS expression was directly dependent on TNFα stimulation. Neuronal cells expressed low basal levels of iNOS as a consequence of cell culture conditions. However, conditioned media and TNFα treatment both stimulated an increase in iNOS protein levels. The mechanism of iNOS-dependent death involves the production of nitric oxide. Although nitric oxide is a relatively weak oxidizing agent, it rapidly reacts with superoxide anion to form the strong oxidizing and protein nitrating agent peroxynitrite (Beckman et al., 1992; Ischiropoulos et al., 1992a,b). Peroxynitrite-dependent apoptosis is a well described phenomenon that occurs in the presence of excess intracellular nitric oxide concentrations (Troy et al., 1996; Estevez et al., 1998; Heneka et al., 1999). The increase in protein nitrotyrosine above the basal in vitro levels observed in the neuronal cultures after treatment with Aβ-stimulated conditioned media from microglia and monocytes is evidence of increased formation of peroxynitrite (Beckman et al., 1992; Keller et al., 1998). This change correlated well with the increase in iNOS expression and was inhibited with specific iNOS inhibitors. These data strongly suggest that the mechanism of apoptosis signaled by conditioned media involves peroxynitrite-mediated oxidative damage.

The present study represents a continuation of our efforts to characterize our in vitro system modeling the microglial-dependent inflammatory changes occurring in AD brains. In this report we have described a bifurcation of Aβ-stimulated signaling events in monocytes. Significantly, this behavior allows for development of pathway-specific therapeutic approaches affecting selective microglial phenotypic changes in response to Aβ-stimulation. We have also shown that increased TNFα production after Aβ-stimulation leads to iNOS-dependent neuronal apoptosis. Microglial-mediated neuron death is not likely to be the sole mechanism of neuronal loss in AD. However, it is encouraging that several markers of “at risk” neurons in AD brains are duplicated in our culture system, suggesting that it is accurately modeling disease events. Elucidation of the microglial-dependent inflammatory changes occurring in AD will continue to offer molecular targets for therapeutic intervention.

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