Introduction

Alzheimer’s disease (AD) is a progressive, irreversible, neurodegenerative disease that affects more than 5.3 million people in the United States [1]. The pathologic processes that underlie this disorder are incompletely understood, however, hypoperfusion/hypoxia is thought to contribute to disease pathogenesis. Hypoxia inducible factor 1-alpha (HIF-1α), a master regulator of cellular responses to hypoxia, is elevated in the microcirculation of AD patients. Cerebral hypoxia is a potent stimulus for vascular activation and angiogenesis. Microvessels isolated from the brains of AD patients express a large number of angiogenic proteins. Despite considerable data in human tissues regarding vascular expression of hypoxia-related angiogenic proteins, there is little information regarding these proteins in the brain vasculature of transgenic AD mice. The objectives of this study were to determine expression of HIF-1α, angiogenic proteins, angiopoietin-2 (Ang-2), and matrix metalloproteinase 2 (MMP2), and survival/apoptotic proteins (Bcl-xL, caspase 3) in the cerebromicrovasculature of AD transgenic mice and to determine the direct effect of hypoxia on cerebral endothelial expression of these proteins in vitro. Cultured brain endothelial cells were subjected to hypoxia for 4-6 h and analyzed by western blot and immunofluorescence. Our results demonstrated that HIF-1α is induced in cultured brain endothelial cells exposed to hypoxia and that expression of Ang-2, MMP2 and caspase 3 was elevated and the anti-apoptotic protein Bcl-xL decreased. Brain sections from AD and control mice showed that HIF-1α, Ang-2, MMP2 and caspase 3 are elevated and Bcl-xL decreased in the microvasculature of AD mice. These data suggest the cerebromicrovasculature is an important target for the effects of hypoxia in the AD brain.

Keywords: Hypoxia, brain microvessels, AD mice, angiogenesis, apoptosis
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between inflammatory and angiogenic processes. Indeed, HIF-1α regulates angiogenesis as well as the transcription of pro-inflammatory genes [21, 22]. Taken together, these data suggest that hypoxia-induced elevation in HIF-1α and angiogenic/inflammatory proteins is a characteristic feature of the microcirculation in human patients with AD.

A growing literature documents vascular abnormalities in transgenic AD mice [23-26]. Studies using in situ brain perfusion and two vascular space markers, inulin and sucrose, indicate that cerebrovascular space and volume are significantly lower in the 3 X Tg-AD mouse models compared to control animals [24]. In AD mice characterized by Aβ and tau accumulation there is an increase in basement membrane thickness surrounding cortical microvessels [25]. More subtle vessel morphological alterations, such as compression and narrowing, that would contribute to localized vessel voids have been detected by time-of-flight angiography [25]. In mouse models of familial AD expressing the Swedish mutation in the Aβ-precursor protein (APPsw) or harboring the Dutch and Iowa vasculotropic amyloid precursor protein mutations, dense Aβ plaques appear to develop initially along blood vessels [26, 27]. Finally, functional vascular abnormalities suggest a perturbation of endothelial metabolic/synthetic properties. In this regard, cerebrovascular autoregulation and the generation of endothelial-derived relaxing factor (EDRF) are both compromised in the brains of AD mice [28, 29].

Despite considerable data in human tissues regarding vascular expression of hypoxia-related inflammatory/angiogenic proteins, there is little information as to these proteins in transgenic mouse models of AD. The objectives of this study are to determine expression of HIF-1α, selected inflammatory/angiogenic proteins (Ang-2, MMP2) as well as survival/apoptotic proteins (Bcl-xL, caspase 3) in the cerebrovascular microvasculature of AD transgenic mice and to determine the direct effect of hypoxia on cerebral endothelial expression of these proteins in vitro.

Materials and methods

Culture and hypoxic treatment of rat brain endothelial cells

Rat brain endothelial cells were obtained from rat brain microvessels, as previously described [31]. The endothelial identity of these cultures was confirmed using antibodies to endothelial cell surface antigen Factor VIII. Endothelial cells used in this study (passages 8 to 12) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 1% glutamine and 1% antibiotics in a humidified 5% CO2 incubator at 37°C. For experiments, rat endothelial cells were plated at a density of 300,000 cells per well onto 6 well plates. The cells were washed three times with Hank’s balanced salt solution (HBSS), the media changed to serum-free DMEM plus 0.1% lactate dehydrogenase and cultures subjected to hypoxia treatment (1% O2) which was achieved by a series of gas exchanges with pure N2.

Western blot analysis

Proteins were extracted from endothelial cell cultures using lysis buffer containing 150 mM sodium chloride, 50 mM Tris, 1% NP-40, 2 mM phenylmethyl sulfonylfluoride. Protein samples (25-30 µg) were mixed with sample buffer (25 mM Tris-HCl, pH6.8, 10% SDS, 30% glycerol, 4% β-mercaptoethanol and 0.02% Bromophenol blue) boiled for 5 min, resolved in 10% SDS-PAGE mini gel and transferred to PVDF membrane. After transfer, the membranes were blocked in Tris-Buffered Saline containing 0.25% Tween-20 (TBST) and 4% nonfat dry milk at room temperature for 2 h, and subsequently incubated with specific antibodies: HIF-1α, Ang-2, MMP2, β-actin, Bcl-xL (ab1, ab65835, ab37150, ab6276, ab98143; Abcam, Cambridge, MA), caspase 3 (sc7148; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 in TBST plus 2% nonfat dry milk at room temperature for 2 h, and subsequently incubated with specific antibodies: HIF-1α, Ang-2, MMP2, β-actin, Bcl-xL (ab1, ab65835, ab37150, ab6276, ab98143; Abcam, Cambridge, MA), caspase 3 (sc7148; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 in TBST plus 2% nonfat dry milk at 4°C overnight. Membranes were washed and bound antibody was detected using peroxidase-conjugated secondary antibodies in TBST (1:5000 dilution) for 1 h. Membranes were washed with TBST for 3 times, developed with chemiluminescent reagents and visualized on film. Average band intensities were measured using Quantity One software (Biorad, Hercules, CA).

Gelatin zymography

Supernatants derived from cultured endothelial cells were centrifuged (200 x g) to remove cellular debris. Samples were mixed with SDS sample buffer without reducing agent (1:1) and
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loaded onto a 10% polyacrylamide gel containing 0.1% gelatin. After electrophoresis, gels were washed in 2.5% Triton X-100 and incubated in low salt collagenase blocking buffer containing 50 mM Tris, 0.2 mM NaCl, 5 mM anhydrous CaCl2 at 37°C for 20 hours. The gels were subsequently stained with 0.5% Coomassie brilliant blue dye and destained with distilled water to visualize the zymogen bands.

Immunofluorescent staining of endothelial cell cultures and mouse brain sections

Endothelial cells were fixed in 3.7% paraformaldehyde for 15 min then incubated with IHC-Tek peroxidase blocking solution at room temperature for 10 min. Cells were incubated at 4°C overnight with primary antibodies for HIF-1α, Ang-2, MMP2, Bcl-xL, and caspase 3, diluted (1:50) in IHC-Tek antibody diluent. Following extensive washing in TBST, cells were incubated with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen, Carlsbad, CA) conjugated secondary antibody (1:400 dilution) at room temperature for 45 min. Following stringent washing with TBST, cells were counterstained with DAPI (4', 6-diamidino-2-phenylindole, dihydrochloride) solution for 8 min. Immunostaining of endothelial cells was viewed using an Olympus IX71 microscope with 40X objective lens and quantified with HAMAMATSU imaging software.

Adult Tg2576 (control) and Tg (APPSWE) 2576 (AD) mice (50-60 weeks of age) were purchased from Taconic (Hudson, NY 12534) and animal procedures were performed in accordance with NIH “Guide for the Care and Use of Laboratory Animals” and Texas Tech University Health Sciences Center Institutional Animal Care and Use Committee (IACUC) guidelines.

Mice were euthanized and brain tissue fixed by transcardial perfusion with 10% neutral buffered formalin (NBF). The brain was removed and 1 mm blocks of tissue from cerebral cortex were post fixed in 10% NBF for additional 12 h and embedded in paraffin. Brain sections were deparaffinized in xylene, hydrated through a graded alcohol series, and then rinsed for 5 min in deionized water. Sections were subjected to heat-induced epitope antigen retrieval, washed with Tris-buffered saline with Tween (TBST) and blocked with 10% donkey serum at room temperature for 2 h. The sections were incubated at 4°C overnight with primary antibodies against HIF-1α, Ang-2, MMP2, Bcl-xL, caspase 3, or the endothelial cell marker Von Willebrand Factor (vWF) in TBS containing 2.5% donkey serum. Sections were then washed, blocked and incubated with appropriate secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594. Sections were incubated with DAPI solution at room temperature for 25 min and viewed using an Olympus IX71 microscope and quantified with HAMAMATSU imaging software.

Statistical analysis

Data from each experiment are expressed as mean ± standard deviation (SD). The one-way ANOVA followed by Bonferroni’s comparison tests were performed for multiple samples. Statistical comparison between AD and control samples as well as between hypoxic and normoxic cell cultures was performed using the Student’s t test. Statistical significance was determined at p<0.05.

Result

HIF-1α is induced in cultured brain endothelial cells exposed to hypoxia and is elevated in brain microvessels from AD mice

Cultured brain endothelial cells were exposed to hypoxia (1% O2) for 6 h and cultures processed for immunoreactivity to the HIF-1α antibody. Endothelial cells maintained under normoxic culture conditions showed little to no detectable reactivity (Figure 1, Top). In contrast, endothelial cells exposed to 6 h of hypoxia displayed strong staining for HIF-1α (Figure 1, Top). Relative quantitation of immunofluorescent reactivity is shown in Figure 1, Bottom.

To further confirm HIF-1α expression, protein lysates isolated from endothelial cell cultures exposed to hypoxia for 4 or 6 h and their corresponding normoxic controls were examined by western blot analysis. The data showed that there was a significant (p<0.01) increase in HIF-1α levels at 4 h of hypoxia and that at 6 h while levels were less than at 4 h, HIF-1α was still significantly (p<0.05) higher than control (Figure 2).

Immunofluorescent examination of tissue sections from the brains of AD mice revealed increased reactivity to the HIF-1α antibody and strong staining compared to vessels from
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Figure 1. (Top) Brain microvessel endothelial cell cultures were exposed to hypoxia (1% O2) for 6 h. Cell cultures were fixed with paraformaldehyde and immunostained with HIF-1α (green) or vWF (red) primary antibody and fluorescence labeled secondary antibody. Nuclear staining was performed using DAPI (blue). (Bottom) The bar graph represents signal intensities normalized with endothelial marker (vWF) and control (C) values set to 1.0. **p<0.01 vs. control.

Figure 2. Brain microvessel endothelial cells cultures were exposed to hypoxia (1% O2) for 4 or 6 h. Protein lysates were separated by SDS-PAGE, transferred to a PVDF membrane and immunoblotted for HIF-1α. Loading equivalency was confirmed using TBP. The lower panel shows band intensities normalized to TBP. Data are mean ± SD of 3 separate experiments. *p<0.05 vs. control (0 h); **p<0.01 vs. control (0 h).

Figure 3. Brain tissue sections from AD mice and control (C) animals were fixed with paraformaldehyde and immunostained with HIF-1α (green) or vWF (red) primary antibody and fluorescence labeled secondary antibody. Nuclear staining was performed with DAPI (blue). (B) The bar graph represents signal intensities normalized with endothelial marker (vWF) and control (C) values set to 1.0. **p<0.01 vs. control.

Control animals (Figure 3). Relative quantitation of immunofluorescent reactivity showed that compared to control vessels from AD mice demonstrated a significantly (p<0.01) higher level of HIF-1α.

Brain endothelial cells exposed to hypoxia up-regulate angiopoietin

Protein lysates isolated from endothelial cell cultures exposed to hypoxia for 4 or 6 h and their corresponding normoxic controls were examined by western blot analysis for Ang-2. The data showed that there was a significant (p<0.05) increase in Ang-2 levels at 4 h of hypoxia. Levels of Ang-2 continued to
Hypoxia increases MMP2 activity and protein levels in cultured endothelial cells

Western blot analysis of protein lysates isolated from endothelial cell cultures exposed to hypoxia for 4 or 6 h and their corresponding normoxic controls showed that hypoxia for 4 or 6 h caused a significant (p<0.05, and p<0.01, respectively) increase in protein levels of MMP2 (Figure 5). MMP-2 activity was also determined in supernatants from endothelial cell culture by gelatin zymography. The data showed that there was a highly significant (p<0.001) increase in MMP2 activity at 4 h of hypoxia and that at 6 h while activity was less than at 4 h, it was still significantly (p<0.01) higher than control (Table 1).

Hypoxia downregulates anti-apoptotic Bcl-xL and upregulates pro-apoptotic caspase 3

Cultured brain endothelial cells were exposed to hypoxia and cultures processed for expression of the anti-apoptotic protein Bcl-xL. After 4 or 6 h of hypoxia western blot analysis revealed a significant (p<0.001) decrease in Bcl-xL expression (Table 2). This was also confirmed by immunofluorescent staining of endothelial cell cultures exposed to hypoxia (1% O2) for 4 or 6 h. Protein lysates were separated by SDS-PAGE, transferred to a PVDF membrane and immunoblotted for Ang-2. Loading equivalency was confirmed using β-actin. The lower panel shows band intensities normalized to β-actin. Data are mean ± SD of 3 separate experiments. *p<0.05 vs. control (0 h); **p<0.01 vs. control (0 h).

Table 1. Activity of MMP2 in cultured endothelial cells

<table>
<thead>
<tr>
<th>Hypoxia (h)</th>
<th>MMP2 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1.00 ± 0.008</td>
</tr>
<tr>
<td>4 h</td>
<td>3.61 ± 0.23***</td>
</tr>
<tr>
<td>6 h</td>
<td>1.75 ± 0.14 **</td>
</tr>
</tbody>
</table>

Supernatants from endothelial cell cultures exposed to hypoxia for 0 (control), 4 or 6 h were processed for gelatin zymography and signal intensity from zymogen bands quantitated. Data are mean signal intensity ± standard deviation from at least 3 separate experiments. **p<0.01, ***p<0.001 vs. control (0 h).
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Table 2. Caspase 3 and Bcl-xL protein levels in cultured endothelial cells

<table>
<thead>
<tr>
<th>Protein</th>
<th>C</th>
<th>4 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-xL</td>
<td>1.00 ± 0.006</td>
<td>0.42 ± 0.049***</td>
<td>0.40 ± 0.072 ***</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>1.00 ± 0.007</td>
<td>4.21 ± 0.12*</td>
<td>11.46 ± 1.17 ***</td>
</tr>
</tbody>
</table>

Protein lysates isolated from endothelial cell cultures exposed to hypoxia for 4 or 6 h and their corresponding normoxic controls were examined by western blot analysis for Bcl-xL and Caspase 3. Data are mean signal intensity ± standard deviation from at least 3 separate experiments. *p<0.05, ***p<0.001 vs. control (0 h).

Expression of Ang-2, MMP2 and caspase 3 are elevated and Bcl-xL decreased in the microvasculature of AD mice

Based on the in vitro experiments which showed that hypoxia affected endothelial expression of Ang-2, MMP2, caspase 3 and Bcl-xL, brain tissue sections from AD transgenic mice and their control littermates were examined by immunofluorescence for expression of these proteins. The results showed that brain vessels from AD mice displayed strong staining for Ang-2, MMP2 and caspase 3 compared to brain vessels from control animals (Figure 8). Relative quantitation of immunofluorescent reactivity...
showed that compared to controls, vessels derived from AD mice demonstrated a significantly (p<0.01) higher level of these proteins (Table 3).

In contrast, immunofluorescent staining for the anti-apoptotic protein Bcl-xL revealed that staining for Bcl-xL was less intense in brain vessels from AD mice compared to vessels from control animals (Figure 8). Relative quantitation of immunofluorescent reactivity showed that compared to controls, vessels derived from AD mice demonstrated a significantly (p<0.01) lower levels of Bcl-xL (Table 3).

**Discussion**

Structural and functional cerebrovascular abnormalities have been identified in AD [32-37]. Increasing evidence points to vascular damage as an early contributor to pathologic processes in the AD brain [38-41]. AD patients with cerebrovascular disease express the clinical symptoms of dementia with fewer AD lesions, suggesting that perturbed blood vessel function synergizes with AD pathology to exacerbate cognitive impairment [42, 43]. Also, epidemiological studies document an association between cardiovascular risk factors and an increased risk of AD [40, 44-48]. Reduction in capillary diameter correlates with Aβ deposition and neurofibrillary tangles as well as with the severity of dementia, as assessed by the Clinical Dementia Rating Scale [49]. Atherosclerosis-induced brain hypoperfusion/hypoxia is thought to contribute to the clinical and pathological manifestations of AD [50, 51]. Neuroimaging and neuropathological studies show that vascular abnormalities and brain hypoperfusion develop early in AD and that the incidence of AD is greatly increased following cerebral ischemia and stroke [2-4].

The mechanisms whereby hypoxia-induced vascular abnormalities contribute to neurodegenerative changes in the AD brain are still unclear. However, a recent study showing that HIF-1α is a modulator of the amyloidogenic processing of APP [52], suggests induction of HIF-1α is a central event in AD pathogenesis. Thrombin, a multifunctional inflammatory protein upregulated in the brain and cerebrovasculature in AD, has been shown to induce HIF-1α in cultured brain endothelial cells [13]. Here we document, using immunofluorescence and western blot, that cultured brain endothelial cells exposed to hypoxia express significantly higher levels of HIF-1α compared to endothelial cell cultures main-
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tained under normoxic culture conditions. Because both hypoxia and inflammatory proteins, such as thrombin, stimulate induction of HIF-1α in brain endothelial cells, HIF-1α may be a focal point for a noxious autocrine loop in this cell type. In the current study, we extend previous observations that HIF-1α is elevated in brain microvessels in human AD samples [13] and show that this transcription factor is overexpressed in the cerebromicrovasculature of the transgenic Tg-2576 AD mouse. Taken together, these data suggest that hypoxia and induction of HIF-1α are important to the evolving pathology of AD.

HIF-1α is a central mediator of downstream physiologic and pathologic processes/factors regulated by hypoxia [53]. In the current study, we show divergent regulation of pro- and anti-apoptotic proteins by hypoxia in cultured brain endothelial cells. We examined key molecular players in apoptosis focusing on proteins that affect both cytosolic and mitochondrial targets. The executioner caspase, caspase 3, is activated downstream by a series of caspases and functions to directly cleave crucial protein substrates resulting in cell death [54]. Bcl-xL, an anti-apoptotic member of Bcl-2 family, prevents translocation of Bax to mitochondria, maintains mitochondrial membrane potential and prevents release of cytochrome c from the mitochondria [55]. Our results show that exposure of cultured brain endothelial cells to 4-6 h of hypoxia increases caspase 3 levels and decreases expression of Bcl-xL. These results are in agreement with published data showing that T cells exposed to hypoxia express low levels of Bcl-xL and that bovine cerebromicrovascular endothelial cells treated with NaCN, to produce hypoxia, show elevated levels of caspase 3 [56, 57].

The notion that pro- and anti-apoptotic mediators are disturbed in cerebromicrovasculature in AD is supported by our immunofluorescent experiments that show AD transgenic mice express higher levels of caspase 3 and lower levels of Bcl-xL when compared to control mice. These results are supported by considerable literature showing dysregulation of pro- and anti-apoptotic mediators in the AD brain [57, 58]. For example, Aβ has been shown to directly activate a number of caspases including caspase-3 and to decrease Bcl-xL [58]. Bcl-xL an anti-apoptotic protein of the Bcl-2 family is expressed at high levels in the adult nervous system. Apoptotic stimuli decrease neuronal Bcl-xL expression while stably overexpressing Bcl-xL in neuroblastoma cells protects them from Aβ neurotoxicity [59].

Although results from both brain endothelial cell cultures and AD mice show that hypoxia supports cell death cascades, hypoxia is also a potent inducer of angiogenesis. In the brain chronic sublethal hypoxia results in release of pro-angiogenic factors from astrocytes and endothelial cells [60]. Therefore, we examined the effects of hypoxia on selected inflammatory/angiogenic proteins (Ang-2, MMP2) in cultured brain endothelial cells as well as expression of these proteins in the cerebrovasculature of AD mice.

Angiopoietins are ligands of the endothelial tyrosine kinase receptor Tie2. Ang-1 is widely expressed in adult tissues and promotes blood vessel maturation and stabilization by inducing Tie2 receptor phosphorylation. In contrast, the antagonistic ligand Ang-2 is upregulated by hypoxia, expressed only at sites of vascular remodeling and plays a crucial role in destabilizing vessels for angiogenesis [61]. The combination of VEGF and angiopoietin-2 promotes more angiogenesis than VEGF alone [62]. Western blot analysis of cultured brain endothelial cells exposed to hypoxia reveals a significant upregulation of this pro-angiogenic mediator. Examination of brain tissue sections from AD transgenic mice shows that vessels stain intensely with the Ang-2 antibody, denoting high level of expression. These data are in agreement with published results documenting overexpression of Ang-2 in the cerebrovasculature of human AD patients [19].

Matrix metalloproteinases are a family of extracellular proteases involved matrix turnover and remodeling. These protease have been shown to enhance angiogenesis by releasing extracellular matrix-bound angiogenic growth factors, by exposing cryptic proangiogenic integrin binding sites in the matrix, and by generating promigratory matrix fragments [63]. A role for MMPs in the pathogenesis of AD is suggested by several studies. MMP9 and TIMP are elevated in postmortem brain tissue from AD patients [64]. We have previously documented upregulation of MMP2 and MMP9 in the isolated microvessels from AD brains. Using multiphoton microscopy in the AD mouse models
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APPsw/P1dE9 and Tg-2576 studies show strong associations among MMP activation, oxidative stress and vascular amyloid deposition [65]. Here we show an overexpression of MMP2 in Tg-2576 mice, compared to control animals. Our data showing increase in MMP2, as well as caspase 3 in response to hypoxia in rodent endothelial cells are congruent with results from a study in human cerebral endothelial cells showing induction of caspase-mediated cell death by MMPs in response to hypoxia-reoxygenation injury [66].

Cerebral hypoxia triggers hypometabolic, cognitive and degenerative changes in the brain and is contributory to the pathology of AD. Indeed, the clinical association of hypoxia and increased incidence of AD has been well described [2-5]. However, the molecular and cellular pathways/processes that are pathologically activated by hypoxia have not been defined. Hypoxia likely facilitates the pathogenesis of AD through multiple mechanisms. Hypoxia-mediated modulation of death/survival signals and expression of inflammatory/angiogenic proteins by brain blood vessels argues for an important role for the cerebrovasculature in pathologic events subsequent to hypoxic injury. In addition, the close proximity of blood vessels to AD lesions supports the notion of intimate vascular-neuronal communication [32]. Whether blocking/blunting angiogenic mediators in AD is beneficial or detrimental is controversial [17, 67, 68]. Despite increases in pro-angiogenic factors no new blood vessel growth is evident in AD; however, epidemiologic studies show that drugs with anti-angiogenic effects are beneficial in AD [69-72]. On the other hand, recent studies suggest that the brain milieu in AD transgenic mice does not support angiogenesis [68]. Further work is urgently needed to determine whether inhibition of hypoxia-mediated effects such as angiogenesis is useful as a therapeutic strategy for AD.

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