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Insulin Degrading Enzyme Is Localized Predominantly at the Cell Surface of Polarized and Unpolarized Human Cerebrovascular Endothelial Cell Cultures

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Insulin degrading enzyme (IDE) is expressed in the brain and may play an important role there in the degradation of the amyloid beta peptide (Aβ). Our results show that cultured human cerebrovascular endothelial cells (HCECs), a primary component of the blood–brain barrier, express IDE and may respond to exposure to low levels of Aβ by upregulating its expression. When radiolabeled Aβ is introduced to the medium of cultured HCECs, it is rapidly degraded to smaller fragments. We believe that this degradation is largely the result of the action of IDE, as it can be substantially blocked by the presence of insulin in the medium, a competitive substrate of IDE. No inhibition is seen when an inhibitor of neprilysin, another protease that may degrade Aβ, is present in the medium. Our evidence suggests that the action of IDE occurs outside the cell, as inhibitors of internalization fail to affect the rate of the observed degradation. Further, our evidence suggests that degradation by IDE occurs on the plasma membrane, as much of the IDE present in HCECs was biotin-labeled by a plasma membrane impermeable reagent. This activity seems to be polarity dependent, as measurement of Aβ degradation by each surface of differentiated HCECs shows greater degradation on the basolateral (brain-facing) surface. Thus, IDE could be an important therapeutic target to decrease the amount of Aβ in the cerebrovasculature.

Key words: Alzheimer's disease; cerebrovascular amyloid angiopathy; insulin; peptidase

The amyloid beta peptide (Aβ) is the major component of amyloid plaques that are key hallmarks of Alzheimer's disease (AD) (Hardy and Selkoe, 2002). This peptide is a cleavage product from a large protein, the amyloid Aβ precursor protein, and accumulates in brain tissues of AD patients in senile plaques. Oligomeric forms of the peptide are very toxic to cells in culture. Aβ deposits in the walls of small arteries of the leptomeninges and cortex cause cerebral amyloid angiopathy (CAA) (Rensink et al., 2003).

In CAA, Aβ deposits are always associated with smooth muscle cells and pericytes around cortical capillaries (de al Torre, 2004). Cerebral hypoperfusion (CBF) in patients with AD is also well established (Bartenstein et al., 1997). The results of a longitudinal study (the Rotterdam Study) demonstrate early decreases in cerebral perfusion before clinical symptoms of AD were seen (Ruitenberg et al., 2005). There have been reports that the density of capillaries is reduced in AD brains (Buee et al., 1997; Suter et al., 2002) although other studies see no change (Bell and Ball, 1981, 1990).

The balance between production and metabolism of the peptide determines the steady-state level of Aβ in the brain. To what extent each process determines the level of Aβ in the brain is unknown. There is recent evidence that the catabolism of Aβ may be involved in the much more common late onset form of AD. Insulin degrading enzyme (IDE), which degrades Aβ as well as insulin, has been implicated as a candidate enzyme responsible for a significant portion of Aβ degradation in

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the brain (Leissring et al., 2003). Several epidemiologic studies implicate the IDE locus in late onset AD and age of disease onset in AD families (Bertram et al., 2000; Ait-Ghezala et al., 2002; Li et al., 2002). Another very recent study implicates IDE in the extent of Aβ deposition in AD patients but not in the risk of developing AD (Blomquist et al., 2005). Some studies, however, have not seen higher risk associated with this region (Abraham et al., 2001; Boussaha et al., 2002; Feuk et al., 2005; Nowotny et al., 2005).

Recent evidence suggests that IDE plays a critical role in the metabolism of Aβ. Two studies have demonstrated that the genetic deletion of IDE in mice leads to excess Aβ in the brain (Farris et al., 2003; Miller et al., 2003). Bigenic mice that produced excess IDE in neurons and over-produced the Swedish mutant form of APP in the brain were shown to develop fewer Aβ containing plaques than do the APP over-producers (Leissring et al., 2003). A very recent study (Caccamo et al., 2005) demonstrates a decreased level of IDE in the hippocampus (a region highly affected in AD) in both a mouse model of AD and human AD patients, whereas the amount of IDE in the cerebellum (an unaffected region) is either unchanged or rises.

IDE is an approximately 110-kDa thiol zinc-metalloendopeptidase located in cytosol, peroxisomes, endosomes, and on the cell surface (Goldfine et al., 1984; Seta and Roth, 1997; Duckworth et al., 1998; Vekrellis et al., 2000). IDE cleaves small proteins of diverse sequence, many of which share a propensity to form beta-pleated sheet-rich amyloid fibrils under certain conditions (Bennett et al., 2000; Kurochkin, 2001). IDE is the major enzyme responsible for insulin degradation in vitro (Duckworth et al., 1998), but the extent to which it mediates insulin catabolism in vivo has been controversial, with some investigators doubting that IDE has any physiologic role in insulin catabolism (Authier et al., 1996).

In view of the evidence discussed above that IDE plays a key role in regulating the level of circulating Aβ and insulin, the subcellular localization of IDE has been confusing. There is now good evidence that IDE is found in the cytoplasm of a variety of cell types (Duckworth et al., 1998). At the c-terminus there is a peroxisomal targeting sequence and IDE has been localized to the peroxisome (Authier et al., 1996). Despite the lack of an obvious N-terminal signal sequence or internal hydrophobic sequences for membrane insertion in the IDE sequence, biochemical studies indicate that a small percentage of IDE is also present at the cell surface of a number of different cell types (Savage et al., 1998; Mukherjee et al., 2000).

Our group has reported evidence that the IDE mRNA and protein are expressed in cultured human cerebral cortical microvascular cells as well as human cerebrovascular endothelial cells (HCECs) in situ (Gao et al., 2004). Another group has also reported that isolated capillaries contain IDE (Morelli et al., 2004). We now demonstrate that the IDE present in HCECs is predominantly located on the plasma membrane and degrades Aβ. In addition, internalization of IDE is not necessary for Aβ degradation. Greater than 50% of IDE is localized to the brain facing side in HCECs that are induced to form tight junctions, a model of the blood–brain barrier (BBB). These results indicate that drugs or gene therapeutic agents that can increase cerebrovascular endothelial IDE levels may be novel treatments for AD and other diseases that produce Aβ-mediated CAA.

MATERIALS AND METHODS

Human cerebrovascular endothelial cells (HCECs) were purchased from Cell Systems (Bellingham, WA). These cerebral cortical cells were originally derived from a rapid autopsy of a 16-year-old male. Some of the original cells were immortalized by a proprietary process to produce the HCECs we employ in this study. Cells were grown in Clonetics EGM-2MV media with added Singlequots supplements, all available from Cambrex (Walkersville, MD). Collagen I and III coated polytetrafluoroethylene (PTFE) cell culture inserts and dishes were purchased from VWR (Westchester, PA). [^{125}I]-Aβ_{1-40} was obtained from Amersham (Piscataway, NJ) or from Peninsula (San Carlos, CA). Protein determinations were made using the DC Protein Assay kit from BioRad (Hercules, CA). Polyacrylamide/bisacrylamide (29:1) solution used for polyacrylamide gels was obtained from BioRad (Hercules, CA). Mouse monoclonal antibody against IDE was obtained from Covance Research Products (Berkeley, CA). Horseradish-peroxidase labeled antibodies were obtained from Rockland Inc. (Gilbertsville, PA) and were detected using Western Lightening enhanced chemiluminescence reagents from Perkin-Elmer (Boston, MA). Biotin labeling was carried out using immobilized neutravidin and EZ link sulfo-NHS-biotin, both from Pierce Biotechnology (Rockford, IL). Unless otherwise indicated, inhibitors and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Culture

For all experiments, HCECs were grown in EGM-2MV media plus Singlequots and containing 5% fetal bovine serum (FBS) and 50 μg/ml gentamycin in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C. For most studies cells were grown to confluence on rat-tail collagen (BD Bioscience, Palo Alto, CA) coated tissue culture dishes. For studies involving cell polarity, however, cells were plated onto collagen I- and II-coated PTFE inserts that were also coated with fibronectin with 0.4 μm pores and cultured as described below.

Aβ Degradation Assay

Cells were grown to confluence. On the day of treatment, media was replaced with fresh media containing either no inhibitor or one or more of the following inhibitors: insulin (100 μM), a competitive inhibitor of IDE (Edland, 2004); phenylmethylsulphonyl fluoride (PMSF, 0.5 mM), an inhibitor of serine proteases (Barr and Warner, 2003); 1,10 phenanthroline (2 mM), a non-competitive inhibitor of IDE (Harada et al., 1993); ethylenediamine tetraacetic acid (EDTA, 5mM), a chelator of divalent ions (Kim et al., 2005); 5,5′-dithiobis

(2-nitrobenzoic acid) (DTNB, 2 mM), a membrane impermeable modifier of thiol groups (Zoccarato et al., 1999); thiorphan (100 nM), a specific neprilysin inhibitor (Roques et al., 1980); phosphoramidon (1 μM), a specific inhibitor of endothelin converting enzyme (Ikegawa et al., 1990); phenylarsine oxide (PAO, 20 μM), an endocytosis inhibitor (Foley et al., 2005); sucrose (0.5 mM), an endocytosis inhibitor (Nieland et al., 2005); or filipin complex IV (Fil, 10 μg/ml), an inhibitor of caveolae mediated endocytosis (Schnitzer et al., 1994). At the time of treatment cells were brought to 10–20 pM with [^{125}I] Aβ_{1-40} and samples were taken at the times specified. TCA precipitation was done and both the pellet and supernatant fractions were measured using a gamma counter. For figures showing specific degradation, the percentage Aβ precipitated in a given sample was determined and subtracted from the percentage of Aβ precipitated after incubation in comparable medium without cells. The Aβ converted to soluble form was regarded as specifically degraded and in some cases was converted into fmol based on specific activity of the radiolabeled Aβ employed.

TCA Precipitation

Samples of cell culture media (100 μl) were taken at the designated times and combined with 30 μl of 20 mg/ml bovine serum albumin and 40 μl of 30% TCA at room temperature. Volumes were adjusted to 200 μl with distilled water and samples were mixed via vortex. After 15 min incubation at −20°C, samples were centrifuged for 5 min at top speed in a tabletop centrifuge. Supernatants and pellets were separated and [^{125}I] was quantified by gamma-counting.

Western Blotting

Protein was measured using the detergent compatible assay. Samples were treated with Laemmli loading buffer (1× final concentration) with 25 mM β-mercaptoethanol, then run onto 10% polyacrylamide gels. Gels were transferred onto a polyvinylidene difluoride (PVDF) membrane, washed, and blocked for 2 hr in PBS with 0.1% Triton X-100 and 1% dry milk. Membranes were incubated overnight with primary antibody and washed three times with PBST. Blots were visualized with the appropriate horseradish peroxidase-conjugated secondary antibody for 2 hr then developed with ECL reagent.

Biotin Labeling

Cells were grown to confluence, lifted and subjected to centrifugation for 5 min at 600 × *g* in a 10 ml conical tube. Cells were washed three times in cold PBS and incubated at room temperature for 30 min in 1 mg/ml Sulfo-NHS-SS-Biotin, a membrane impermeable biotinylation reagent. After labeling, cells were washed twice with PBS containing 0.1% Triton X-100. Cell lysates were incubated overnight at 4°C with a quarter of the volume of neutravidin-coated agarose beads, after which the avidin beads were separated and washed. The avidin-bound and unbound fractions were separately subjected to SDS-PAGE and analyzed by Western blotting with anti-beta actin antibody.

HCEC Polarity Experiments

HCECs were grown to confluence on 30 mm PTFE membranes, with 0.4 μm pores, placed in the chambers of divided-compartment 12-well plates. Membranes were purchased precoated with collagen I and III and were given an additional coat with fibronectin. To promote tight junction formation, confluent cells were maintained in the presence of 250 mM cAMP and 17.5 mM 4-(butoxy-4-methoxybenzyl)-2-imidazolidinone for an additional 24 hr before use (Rubin et al., 1991; Davies et al., 2000). FITC-dextran (4 kDa) was added to the upper chamber to a concentration of 1 mg/ml and medium from both chambers was measured at 0, 3, and 18 hr. Concentration of FITC-dextran from each sample was determined using a standard curve of FITC-dextran emission at 515 nm after excitation at 488 nm. To assess the permeability of the barrier system to Aβ, [^{125}I]-Aβ_{1-40} was added to the top chamber to a concentration of 100 pM and samples were taken at 0, 3, and 18 hr from both the top and bottom chambers.

Statistical Analysis

Student's *t*-test was used for single comparison of means and for multiple comparisons of differences in means, one-way ANOVA followed by Tukey-Kramer post-hoc analysis were used. Experiments were carried out at least three times in all cases.

RESULTS

Understanding the ways in which Aβ is degraded biologically is of great importance in developing therapies that target this pathway. Only a few enzymes are known that possess a meaningfully selective affinity for Aβ and that may be suspected of being part of the natural degradative pathways. These enzymes include neprilysin, the endothelin converting enzyme, and insulin degrading enzyme (IDE). We wished to assess whether IDE might play a significant role in degradation taking place in the blood–brain barrier.

We sought to demonstrate whether monocultured HCECs were fully capable of degrading Aβ. When [^{125}I]-Aβ was added to the medium of growing HCECs, a time-dependent reduction is observed in the fraction of Aβ precipitable with trichloroacetic acid. This corresponds to a reduction in the average length of the Aβ molecule due to degradation by some protease (Fig. 1A). This degradation is not observed when Aβ is incubated in medium that does not contain cells (compare to data in Fig. 4) nor is it observed in conditioned medium from HCECs (data not shown). Addition of insulin, a preferred substrate for IDE (Edland, 2004), to the medium results in a significant reduction in the rate of Aβ degradation. In addition, phosphoramidon, an inhibitor selective for endothelin converting enzyme (Ikegawa et al., 1990) was found to be effective in blocking much of the remaining degradative activity (Fig. 1B). We chose, however, to focus on the activity of the IDE present. In contrast, addition of thiorphan, used at levels capable of inhibiting neprilysin (Dolev and Michaelson,

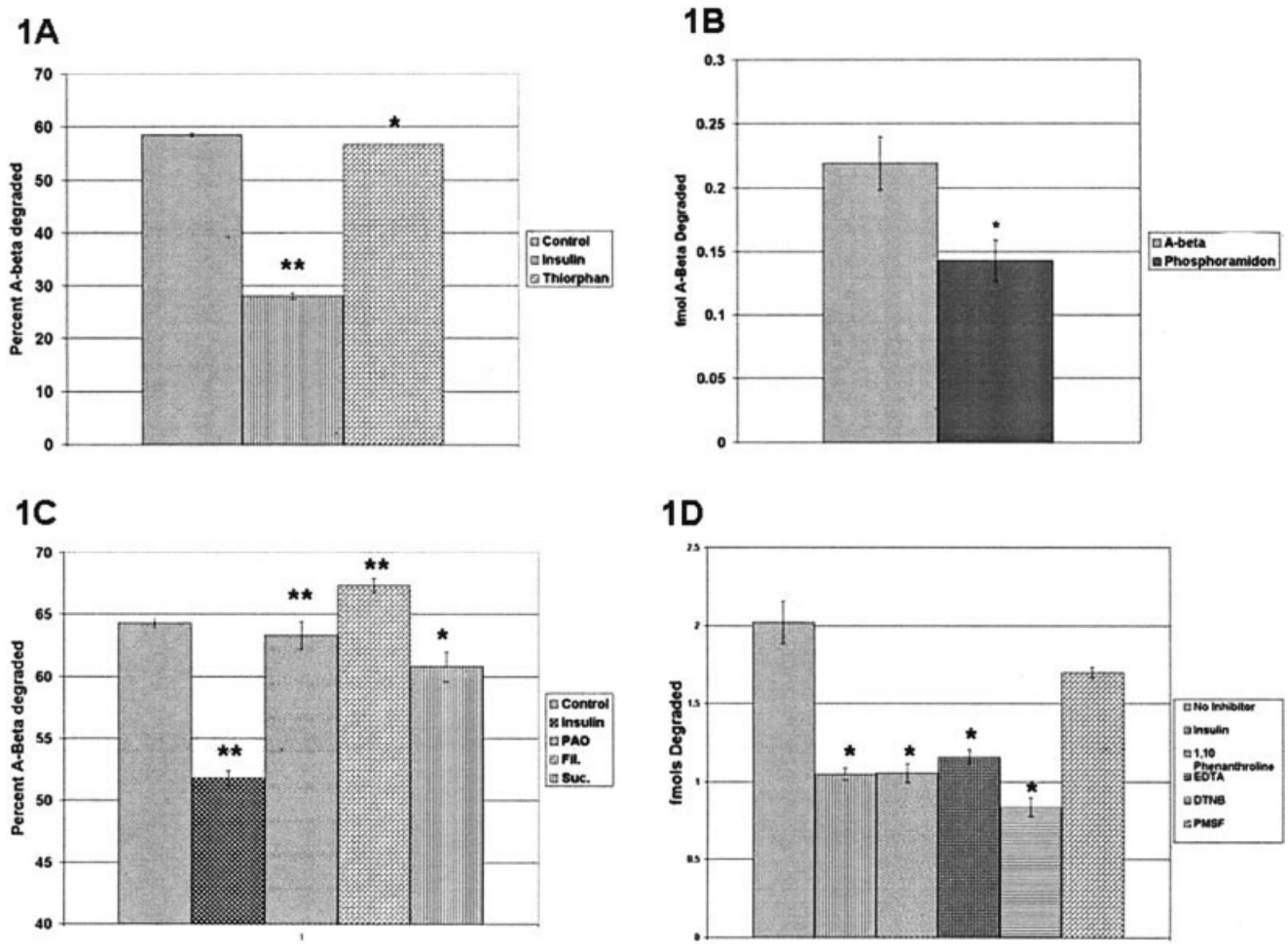

Fig. 1. Degradation of Aβ by HCECs is attenuated by insulin and phosphoramidon, but by not thiorphan or inhibitors of endocytosis. HCECs were grown to confluence, then pretreated with inhibitors of specific proteases or endocytotic pathways (as indicated), or were left untreated (control) for 30 min in the 37°C incubator. Cells were then coincubated with [^{125}I]-labeled Aβ and various inhibitors for 24 hr and samples of media were taken. **A:** Cells were coincubated with media containing [^{125}I]-labeled Aβ and either untreated (control), treated with insulin (100 μM), a competitive substrate of IDE, or thiorphan (100 nM), a specific inhibitor of neprilysin. Values are means ± SEM; $*P < 0.01$, $**P < 0.001$. **B:** Cells were coincubated with media containing [^{125}I]-labeled Aβ and endothelin converting enzyme inhibitor, phosphoramidon (1 μM). Values are means ± SEM; $*P < 0.02$. **C:** Cells were coincubated with media containing [^{125}I]- labeled Aβ and either insulin or inhibitors of receptor-mediated endocytosis, phenylarsine oxide (PAO, 20 μM) and sucrose (0.5 M) or clathrin-mediated endocytosis inhibitor, filipin complex (Fil., 10 μg/l). Values are means ±SEM; $*P < 0.01$, $**P < 0.001$. **D:** Cells were coincubated with media containing [^{125}I]-labeled Aβ and insulin or one of the following: zinc chelating agents 1,10 phenanthroline (20 μM) and EDTA (1 mM), the membrane impermeable thiol-protease inhibitor dithiobisnitrobenzoate, DNTB, (100 μM) and PMSF (100 μM) for 24 hr. Values are means ± SEM; $*P < 0.05$.

2004), fails to even modestly alter the rate at which degradation occurs (Fig. 1A).

These data indicate that IDE is responsible for a significant portion of the Aβ degradation. This degradation is not dependent on internalization as neither sucrose, phenylarsine oxate (PAO) (inhibitors of endocytosis (Neiland et al., 2005; Foley et al., 2005)), nor filipin complex (an inhibitor specific for caveolin-dependent endocytosis (Inal et al., 2005)) are able to substantially reduce the rate of degradation (Fig. 1C). The Zn(2+)-specific chelator 1,10 phenanthroline and the divalent metal chelator EDTA reduced Aβ degradation to the same extent as insulin (Fig. 1D) (Harada et al., 1993; Kim et al., 2005). The same effect was seen with dithiobisnitrobenzoate (DNTB), a membrane impermeable inhibitor of proteases requiring thiols for activity, such as IDE (Zarracato et al., 1999). No significant reduction in Aβ degradation was seen in the presence of the serine protease inhibitor, PMSF (Barr and Warner, 2003).

As these results are consistent with IDE directly degrading soluble Aβ on the surface of the cells, we set out to determine whether, and to what extent, IDE exists on the surface of HCECs. To identify only cell-surface proteins, we employed NHS-SS-biotin, which is

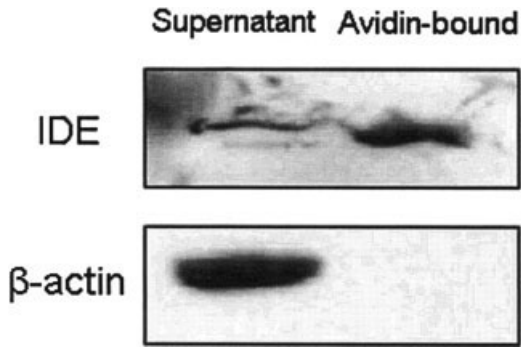

Fig. 2. IDE is mostly present on the plasma membrane of HCECs. HCECs were grown to confluence, washed with cold PBS, incubated at room temperature for 30 min in 1 mg/ml Sulfo-NHS-SS-Biotin, a membrane impermeable biotinylation reagent. After labeling, excess reagent was washed away and cells were lysed in a buffer containing Triton X-100 (0.1%). Cell lysates were incubated overnight at 4°C with a slurry of neutravidin-coated agarose beads, after which the avidin beads were separated and washed. The avidin-bound and unbound fractions were subjected separately to SDS-PAGE and assayed for IDE and for β-actin by Western blot analysis.

a hydrophilic reagent that is unable to pass through the plasma membrane and binds strongly to avidin. Consequently, it reacts solely with proteins attached to the extracellular portion of the cell membrane and labels them with a biotin marker, whereas internal proteins remain unlabeled. Labeling of HCECs was followed by precipitation with beads coated with neutravidin, a neutral pKa, non-glycoprotein form of avidin. This allowed for the separation of externally present proteins (biotin labeled) from the internal proteins of the HCECs (non-biotin labeled). To determine whether IDE was present at the cell surface, we used Western blotting to analyze the levels of IDE in each of the two isolated fractions. Likewise, for comparison, we analyzed the levels of β-actin, an internal protein control. In Figure 2 it can be seen that HCEC IDE is primarily found in the biotin-labeled (precipitated) fraction, whereas β-actin is almost completely found in the non-labeled (supernatant) fraction. This labeling shows that IDE is primarily present on the surface of HCECs.

We further wished to investigate the manner in which HCECs may degrade Aβ. We utilized a model in which the brain-facing and blood-facing surfaces of the cell could be isolated and individually examined experimentally as first described by Rubin et al. (1991). To segregate the two chambers, HCECs were induced to form a model of the blood–brain barrier as described in Materials and Methods. To test the effectiveness of the barrier formed, either 4kDa FITC-conjugated dextran or [^{125}I]-Aβ was added to one chamber or the other of the barrier model and samples were taken from both chambers over time to determine the rate of diffusion between the sides of the membrane. Only the TCA-precipitated form of Aβ was counted as it was found that degradation products crossed the barrier more freely than

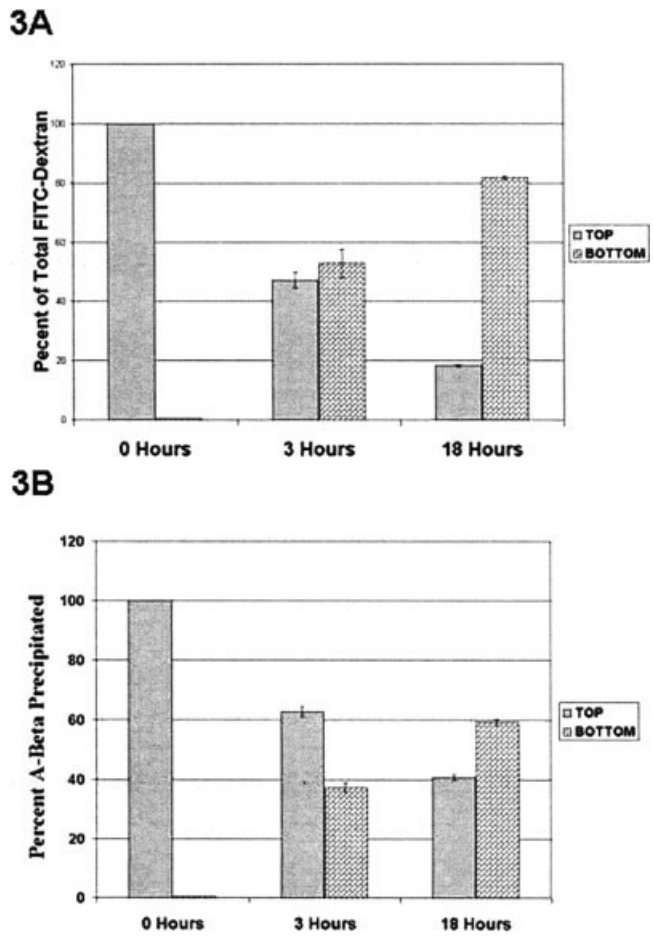

Fig. 3. HCECs can form a semi-permeable barrier against diffusion by 4 kDa FITC-Dextran and Aβ. HCECs were grown to confluence on PTFE membranes that were suspended in the chambers of divided-compartment 12-well plates. To promote tight junction formation, confluent cells were maintained in the presence of 250 mM cAMP and 17.5 mM 4-(Butoxy-4-methoxybenzyl)-2-imidazolidinone for an additional 24 hr before use. **A:** After tight junction formation, 4 kDa fluorescein isothiocyanate-conjugated dextran (FITC-dextran) was added to the upper chamber to a final concentration of 1 mg/ml and media samples were taken at 0, 3, and 18 hr from the top and bottom chambers. Concentration of the FITC-dextran from each sample was determined using a standard curve of FITC-dextran emission at 515 nm after excitation at 488 nm. **B:** After tight junction formation, Aβ was added to the top chamber to a final concentration of 100 pM and samples were taken at 0, 3, and 18 hr from the top and bottom chambers. Relative Aβ concentration was determined by TCA precipitation and gamma counting each sample, then expressing the concentration as a percentage relative to the total undegraded Aβ present in the well at the time the sample was taken.

intact peptide. As can be seen in Figure 3A, FITC-dextran added to the top chamber diffused slowly, reaching equilibrium across the chambers after 18 hr. More importantly, Aβ added to the top chamber diffused more slowly than FITC-dextran, failing to diffuse to equilibrium even after 18 hr (Fig. 3B). Similar results were seen when diffusion was monitored from bottom chamber to

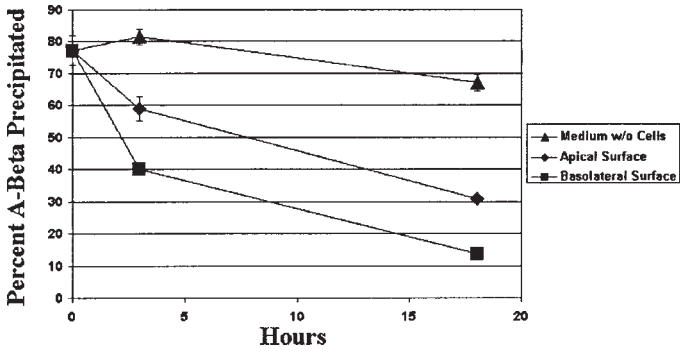

Fig. 4. HCECs degrade Aβ preferentially on the basolateral (brain-facing) surface. HCECs were grown to confluence on PTFE membranes as described in the previous Figure 3. [^{125}I] Aβ was added to either the top or bottom chamber and media samples were taken at 0, 3, and 18 hr from top and bottom chambers. Media samples were precipitated with TCA and the percentage of precipitated-to-total counts (corresponding to undegraded Aβ) was determined by gamma counting. As a control, Aβ was incubated without cells, in the presence of growth medium.

top. These results indicate that this barrier is able to block diffusion of 4 kDa dextran for at least 3 hr, and is an even better barrier for undegraded Aβ.

We next wished to assess whether there are differences in the two surfaces of the HCECs grown across a membrane barrier with respect to degradation of Aβ. The top and bottom surfaces have been shown to correspond to the blood facing and brain facing sides of the blood–brain barrier respectively (Rubin and Staddon, 1999). Cells were differentiated as before and 40 nM [^{125}I]-Aβ was added to either the top or the bottom chamber. Degradation of Aβ was monitored with TCA precipitation and gamma counting of the supernatant and pellet fractions. Although rapid degradation of Aβ seen as a conversion from precipitated to non-precipitated counts, was seen in both chambers, more Aβ degradation was observed in the lower chamber than in the upper (Fig. 4). A very similar result was obtained when the amount of insulin-inhibited Aβ degradation was determined on both surfaces of the HCECs (data not shown). Thus, there is more IDE present on the basolateral than the apical surface of the HCECs.

DISCUSSION

We have shown that cultured HCECs, a model for the human blood–brain barrier, contain IDE expressed on both apical (blood facing) and basolateral (brain facing) surfaces. Much of the detectable IDE is present on the cell surface, as indicated by our ability to label the protein with a reagent that cannot cross the plasma membrane. As expected for an enzyme expressed at the cell surface, IDE can degrade its substrate, Aβ, in the presence of several reagents that inhibit internalization. Our data indicate that essentially all Aβ degradation by these cells can be accounted for by the action of surface enzymes including IDE and endothelin converting enzyme.

The finding that IDE is largely expressed at the cell surface is in contrast with the results of other groups who have examined a variety of other cell types (Duckworth et al., 1998). In liver, IDE seems to be present in the cytosol and in peroxisomes, which is consistent with it containing a peroxisome localization signal (Akiyama et al., 1988; Morita et al., 2000). Recently a novel translational isoform that contains a mitochondrial localization signal and is targeted to that organelle has been reported (Leissring et al., 2004). Other investigators have reported the presence of IDE on the plasma membrane or secreted into the extracellular medium surrounding the cells (Vekrellis et al., 2000). The proportion of the enzyme found on the surface in these studies seems to be quite small compared with that found in other compartments.

Our finding that there is a high percentage of IDE on the extracellular side of the HCEC plasma membrane is difficult to explain based on its amino acid sequence. It contains no signal sequence at its N-terminus, and there is no 20–22 stretch of hydrophobic amino acids within its open reading frame. One possible explanation is that there is an undiscovered alternate exon that causes the protein to be inserted into the ER and transported to the plasma membrane. Another alternative possible mechanism to transport IDE out of the cell is through an ATP dependent transport system. Other large polypeptides including fibroblast growth factor and interleukin I have been shown to be transported outside the cell by this mechanism (Kuchler and Thorner, 1992). Further experiments are necessary to determine what mechanism is being employed in the case of IDE.

Another unanswered question is the mechanism by which IDE is tightly anchored to the plasma membrane. Our data indicates that IDE behaves like a membrane protein, as indicated by its association with membrane pellets (data not shown). This is despite the fact that there is no indication of either a hydrophobic sequence or a consensus sequence to which either a fatty acid or glycosylphosphatidylinositol molecule could be attached in the protein sequence. Again the possibility remains that a yet undiscovered alternative exon contains either of the above mentioned sequences. It is also possible that the protein forms a three-dimensional structure allowing it to be partially embedded in the plasma membrane outer lipid bilayer.

It has been determined that insulin degradation via IDE is an insulin receptor-dependent process, suggesting a physical interaction between the two proteins. Vascular endothelial cells have insulin receptors and insulin enters the brain via an insulin receptor-dependent transcytotic transport (Banks et al., 1997). An important but unanswered question is whether the degradation of the other substrates of IDE including Aβ, are also insulin receptor-dependent processes. If this were true then the physical interaction between IDE and the insulin receptor would produce a more active enzyme. If not then it would

indicate that insulin binding to its receptor serves to bring it into proximity with IDE, allowing it to be cleaved. Other peptides with no affinity to the insulin receptor would have no greater access to IDE than if the insulin receptors were not present.

The finding that IDE is found on both the apical (blood facing) and basolateral (brain facing) compartments of the HCECs suggests that the enzyme could play distinct roles on the two surfaces of endothelial cells that comprise the blood–brain barrier. On the apical side, it would likely metabolize insulin, and because 20% of the resting blood flows through the brain at any given time, it could play a key role in insulin degradation. In contrast, even during sleep and between meals, when insulin plasma concentrations are lower (about 100 nM), IDE would very likely not be involved in Aβ catabolism, due to competitive inhibition by insulin.

The situation is very different at the basolateral surface. The concentration of insulin is at least an order of magnitude lower than in the plasma. In contrast, the concentration of Aβ species is at least 100-fold greater (Savage et al., 1998; Shiiki et al., 2004). Therefore IDE is likely to be a key enzyme in reducing Aβ concentration in the extracellular fluid in the CSF as well as in the extracellular fluid surrounding the vascular cells. This reduction may be very important in maintaining the Aβ concentration below the level at which it begins to oligomerize, thereby forming toxic species that can damage the vasculature. IDE can only degrade monomeric Aβ (Qui et al., 1998), but by doing so it can shift the equilibrium away from the toxic oligomeric species (Lambert et al., 1998) as well as from Aβ fibers contained in plaques and vascular deposits.

Based on evidence coming from Aβ vaccine development that all the Aβ species in the body including the Aβ in plaques are in equilibrium (Schenk, 2002), we would postulate that methods that would elevate IDE in cerebrovascular endothelial cells would have the effect of reducing both cerebrovascular and parenchymal plaques, as well as toxic oligomeric intermediates. Two patients who were given the Aβ vaccine developed by Elan, and who later died showed a marked reduction in parenchymal plaque burden, but no decrease in vascular plaques (Nicoll et al., 2003; Ferrer et al., 2004). This finding suggests that a drug or gene therapy that raises the concentration of IDE in the cerebrovascular endothelium may be very useful in combination with a vaccine. Victims of CAA will be another likely group of patients to benefit from this treatment. The advantage of drugs or gene therapies that target the cerebrovascular endothelial cells instead of the brain, is that these agents do not have to cross the intact blood–brain barrier.

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