The α5β1 Integrin Mediates Elimination of Amyloid-β Peptide and Protects Against Apoptosis

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Abstract. The amyloid-β peptide (Aβ) can mediate cell attachment by binding to β1 integrins through an arg-his-asp sequence. We show here that the α5β1 integrin, a fibronectin receptor, is an efficient binder of Aβ, and mediates cell attachment to nonfibrillar Aβ. Cells engineered to express α5β1 internalized and degraded more added Aβ1-40 than did α5β1-negative control cells. Deposition of an insoluble Aβ1-40 matrix around the α5β1-expressing cells was reduced, and the cells showed less apoptosis than the control cells. Thus, the α5β1 integrin may protect against Aβ deposition and toxicity, which is a course of Alzheimer’s disease lesions.

INTIGRIN-mediated cell adhesion is necessary for the survival of many types of cells, and loss of adhesion causes apoptosis (reviewed in Frisch and Ruoslahti, 1997). The α5β1 integrin may have a particularly prominent antiapoptotic effect because α5β1 is the only integrin that protects cells from apoptosis in serum-free cultures (Zhang et al., 1995; O’Brien et al., 1996). α5β1-mediated adhesion upregulates the antiapoptosis protein Bel-2 (Zhang et al., 1995), and α5β1 is one of a few integrins that activates the signaling protein Shc (Wary et al., 1996). These signaling events may partly explain its antiapoptotic effects.

β1 integrins have been shown to mediate cell adhesion to the amyloid beta (Aβ)1 protein, and α5β1 has been proposed to be the integrin responsible for the Aβ binding (Ghiso et al., 1992). The amino acid sequence arg-his-aspartic acid (RHD) has been pinpointed as the integrin recognition site in Aβ (Ghiso et al., 1992; Sabo et al., 1995). This sequence resembles the general integrin recognition sequence RGD present in many extracellular matrix proteins (Ruoslahti, 1996).

Aβ is a 39–42 amino acid protein derived from proteolytic cleavage of a larger membrane-spanning glycoprotein, the amyloid precursor protein (APP; Kang et al., 1987). Aβ forms fibrillar aggregates that can cause cell death by apoptosis (Loo et al., 1993; Pike et al., 1993; Lorenzo and Yanker, 1994). Enhanced deposition of Aβ matrix within the cortex, hippocampus, and vasculature of the brain correlates with neuronal cell death and ultimately dementia in Alzheimer’s disease (AD; reviewed by Selkoe, 1994). Two predominant forms of Aβ (1–40 and 1–42) exist in AD that differ by two amino acid residues at the hydrophobic COOH terminus, a domain that is required for nucleation-dependent fibril formation (Jarret et al., 1993). The Aβ1-40 form has a slower rate of fibril formation in vitro than the Aβ1-42 form (Jarret et al., 1993).

There is evidence for three mechanisms of Aβ accumulation: overproduction of Aβ, production of longer forms of Aβ (which aggregate more), and impaired clearance of Aβ. The clearance pathways for fibrillar and soluble Aβ are incompletely known. Two cell surface receptors are known to bind Aβ. The scavenger receptor present on glial cells binds specifically to fibrillar Aβ, and appears to mediate clearance of small fibrillar Aβ aggregates in vitro (Paresce et al., 1996; Khoury et al., 1996). The receptor for advanced glycation end products binds both the soluble and fibrillar forms of Aβ, and may mediate some of the cytotoxic effects of fibrillar Aβ (Yan et al., 1996).

Because α5β1 may also be an Aβ receptor, and because α5β1 and Aβ have apparently contrasting effects on apoptosis, we sought to determine whether α5β1 is indeed an Aβ-binding integrin and, if so, what effect it might have on the metabolism of Aβ and on cell survival. We show here that nonfibrillar Aβ binds to the α5β1 integrin, and that

1. Abbreviations used in this paper: Aβ, amyloid β peptide; AD, Alzheimer’s disease, LDH, lactate dehydrogenase; RHD, arg-his-aspartic acid.
this interaction promotes clearance of Aβ by cultured cells, reducing the formation of an insoluble Aβ fibrillar matrix and countering the toxic effects of the Aβ matrix. These results suggest a new function for α5β1 as a binder of Aβ and a regulator of brain cell survival.

Materials and Methods

Cells

The human neuroblastoma cell line (IMR-32) was obtained from the American Type Culture Collection (Rockville, MD). The CHO-B2 cells deficient in α5β1 were from Dr. Rudolf Juliano (School of Medicine, University of North Carolina, Chapel Hill, NC; Schreiner et al., 1989). All cells were maintained in α-MEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS and glutamine/pen-strep (Irvine Scientific, Santa Ana, CA). G418 (GIBCO BRL, Gaithersburg, MD) was added to the media of transfected cells at a concentration of 250 μg/ml.

Reagents

Amyloid beta 1-40 peptide (Aβ) was synthesized as previously described (Nordstedt et al., 1994). Aβ was also purchased from a commercial source (Synthetic Amyloid Beta peptide 1–40; Bachem, Torrance, CA). Aβ1-40 from both sources was examined for cell adhesion activity. Two out of the three Bachem lots tested showed active adhesion activity (lots z057 and z056), while lot z032 was not active. For water-free storage to prevent aggregation of Aβ into its fibrillar form, the peptide was dissolved and stored in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Fluka Chemika, Neu-Ulm, Switzerland). Before use, the peptide was lyophilized from HFIP, dissolved in sterile distilled water at 1 mg/ml, and tested immediately. The control peptide, Aβ40-1, was purchased from Bachem, solubilized in water at 1 mg/ml, and tested immediately. Fibronecitin was purchased from Chemicon International, Inc. (Temecula, CA), and vitronectin was purified as described (Yatohgo et al., 1988). Purified anti-human α5 integrin monoclonal antibody (P1D6; Calbiochem-Novabiochem Corp., La Jolla, CA; Wayner et al., 1988) and purified mouse IgG (Sigma Chemical Co.) were used at a concentration of 50 μg/ml.

Transfection

The CHO-B2/α5β1, CHO-B2/α5β1-, and IMR-32/α5β1- cells were generated by introducing cDNAs coding for the α5 and αv integrin subunits into α5β1-deficient CHO-B2 and IMR-32 cells (Schreiner et al., 1989; Bauer et al., 1992; Zhang et al., 1993, 1995). Transfectants expressing the integrin were cloned and expanded (Zhang et al., 1993; Zhang et al., 1995). CHO-B2 and IMR-32 control cells received the empty vector.

Integrin Analysis

Integrin expression of IMR-32 and CHO transfectants was analyzed by FACS using monoclonal antibodies against human α5 (P1D6), αv (L200), and β1 (P4C10). FITC-conjugated goat anti-mouse antibody (Sigma Chemical Co.) was used as the secondary antibody. The same integrin antibodies were used to block integrin function in other experiments.

Cell Adhesion to Nonfibrillar Aβ1-40

The cell attachment assay and the use of antibodies and peptides as inhibitors of adhesion have been described previously (Zhang et al., 1993; Matter and Laurie, 1994). Microtiter plates coated overnight at room temperature with nonfibrillar Aβ1-40 peptide, control Aβ40-1 peptide, or fibronecitin were blocked with 1% BSA for 30 min at room temperature, the wells were rinsed once with PBS (pH 7.4), and cells were subsequently added (2 x 10⁵ cells/well) in serum-free media and incubated for 60 min (37°C). Inhibition studies were performed by preincubating cells with antibody for 30 min (37°C; gentle agitation every 10 min), and then cells including antibodies were added to the coated wells. After a 60-min incubation at 37°C, plates were gently washed four times with PBS, fixed with 1% glutaraldehyde (Sigma Chemical Co.), PBS-washed once, stained with 0.5% crystal violet, 20% MEOH, washed under running distilled water, solubilized in 0.1 N sodium citrate, 50% ETOH, and read on an ELISA plate reader (Molecular Devices Corp., Sunnyvale, CA) using the 590-nm filter.

Adhesion assays with fibrillar Aβ1-40 were performed as above. Before the adhesion assay, soluble Aβ1-40 was incubated at 4°C for 96 h to allow self-aggregation of Aβ1-40 into its fibrillar form (Jarret et al., 1993). Coating efficiency was measured by coating microtiter wells with either soluble [125I]Aβ1-40 or preaggregated [125I]Aβ1-40 at room temperature overnight. Nonbound peptide solution was removed, and the well and the nonbound peptide solution were counted. Both forms of Aβ1-40 bound to the wells with an efficiency of ~70%.

Immunostaining of Aβ Fibrillar Matrix

Cells were plated on four-well Permanox™ plastic slides (Nunc Inc., Naperville, IL) at 50,000 cells/well. 6 h after plating, the media was replaced with media containing Aβ1-40 peptide (100 μg/ml) and incubated for 72 h at 37°C. The cultures were washed with PBS and fixed in PBS containing 3.7% paraformaldehyde and 10 mM sucrose, pHE 7.4, for 30 min at room temperature. The cultures were then blocked with 1% BSA/PBS and stained with a polycyonal rabbit anti-human Aβ1-40 peptide antibody (Chemicon International, Inc.) for 2 h, followed by goat anti-rabbit FITC-labeled IgG (Sigma Chemical Co.) secondary antibody. After antibody treatment, coverslips were mounted with Vectashield mounting medium (Vector Labs., Inc., Burlingame, CA) and analyzed under a fluorescent confocal microscope.

Analysis of Aβ in Matrix Deposition with Radiolabeled [125I]Aβ1-40

[125I]Aβ1-40 peptide was purchased as a lyophilized powder (25 μCi) from Nycoderm Amersham, Inc. (Princeton, NJ). The powder was solubilized in sterile water and immediately added to 24-well culture dishes at a concentration of 2 ng/well. The specific activity of the [125I]Aβ1-40 peptide was 2 x 10⁶ cpm/μg.

Solvabilization of Aβ was analyzed using [125I]Aβ1-40 peptide as described previously for fibronectin matrix assembly (McCown-Longo and Mosher, 1985; Morla and Rusloahiti, 1992). Cells were plated at 10⁵ cells/well (IMR variants) or 0.5 x 10⁵ cells/well (CHO variants) into 24-well tissue culture plates in media containing 10% serum. Media was replaced 6 h after plating with media containing [125I]Aβ1-40 and 10% serum. Cells were cultured at 37°C for 72 h. The media was removed, and the wells were washed three times with PBS, and 5 x 10⁶ SDS sample buffer (0.5M Tris pH 6.8, glycerol, 10% SDS, 0.5% bromophenol blue) was used to solubilize the [125I]Aβ matrix in each well.

For antibody inhibition experiments, cells were plated as above. 6 h after plating, the media was replaced with media containing the appropriate antibody and 10% serum. [125I]Aβ1-40 peptide (2 ng/well) was added to the antibody-containing media and incubated for 72 h at 37°C. The cells were then processed as above.

Internalization and Degradation of [125I]Soluble Aβ1-40

Internalization of Aβ1-40 added to cell layers was measured as described (Duckworth et al., 1972; McDermott and Gibson, 1997). Subconfluent cells were trypsinized and plated onto 24-well plates. Media was replaced 6 h after plating with [125I]Aβ1-40 (2 ng/ml). The cells were incubated for 1 h with [125I]Aβ1-40, the media was removed, cells were washed five times with PBS, and serum-containing media containing no Aβ1-40 was added. The cells were cultured for 1 to 12 h at 37°C, washed three times with PBS, detached by EDTA, washed twice with PBS, and lysed in 100 μl of 1% NP40 buffer for 10 min at 4°C, and lysate-analyzed for radioactivity. For TCA precipitations, the cells were cultured for 72 h with [125I]Aβ1-40 at 37°C, washed three times with PBS, detached by EDTA, washed twice with PBS, and lysed in 100 μl of 1% NP40 buffer for 10 min at 4°C. BSA/PBS (100 μl, 1%) was added to the samples, the samples were vortexed, and 1.6 ml of TCA (12.5% wt/vol) was added with vortexing. The samples were centrifuged at 2,000 rpm for 10 min at 4°C, and the supernatant and pellet were collected for radioactive counting.

Secretion of [125I]-Labeled Aβ1-40

Subconfluent cells were detached with trypsin, washed once with media, and plated at 10⁵ cells/ml in 24-well plates. 6 h after plating, media was replaced with 2 ng/ml of [125I]Aβ1-40 in serum-containing media and incubated for 1 h at 37°C. The radiolabeled media was removed, and cells were
Figure 1. Integrins mediate CHO cell adhesion to nonfibrillar Aβ. Adhesion of CHO cells to coated Aβ1-40 was measured. The cells were transfected with human α5 or αv integrin subunit cDNA. (A) The cells were seeded onto various concentrations of coated Aβ1-40. (B) Cells were plated onto Aβ1-40 coated in its soluble form, Aβ1-40 coated after self-aggregation into its fibrillar form, or on the control peptide Aβ40-1. (C) Cells were preincubated with either the blocking monoclonal antibodies to human α5 (P1D6), human αv (L230), the integrin-binding peptideGRGDSP, or the control peptide GREGESP, and then seeded onto coated soluble Aβ1-40. After a 60-min incubation at 37°C, attached cells were quantitated. Values represent the mean ± SD; n = 9.
washed five times in PBS before serum-containing media containing no Aβ was added to each well. At designated time points, 100 μl of media was collected, and [125I] was measured.

**Apoptosis and Cell Viability Assays**

The apoptotic effect of fibrillar Aβ was determined using the Apoptag Plus In Situ Apoptosis Kit™ (Oncor, Inc., Gaithersburg, MD) that detects the 3'-OH region of cleaved DNA. Cells were plated on eight-chamber tissue culture glass slides (Miles Scientific Laboratories, Inc., Naperville, IL), and 6 h after plating the media was replaced with media containing either Aβ1-40 peptide (50 μg/ml) or Aβ40-1 control peptide (50 μg/ml) and 10% serum. Cells were cultured for 72 h at 37°C, and were then fixed in a solution containing 3.7% paraformaldehyde, 10 mM sucrose in PBS for 30 min at room temperature. Cells were stained following kit protocol, counterstained with propidium iodide/antifade solution (Oncor, Inc.), mounted, and viewed under a confocal microscope.

To measure apoptosis by nuclear fragmentation, cells were plated in wells coated with either 50 μg/ml of fibronectin, vitronectin, or Aβ1-40 for 72 h in serum-free medium. Attached and floating cells were then collected by centrifugation, washed once with PBS, fixed with 3.7% paraformaldehyde for 10 min at room temperature, and stained with 0.1 μg of 4′, 6-diamidino-2-phenylindole (DAPI) per ml in PBS. The stained cells were washed three times with PBS and mounted onto slides for analysis under a fluorescence microscope (Zhang et al., 1995).

Cell viability was assessed in several assays. The ability of cells to take up acidine orange/ethidium bromide was measured as described (Cotter and Martin, 1996). In brief, the assay was performed in 96-well tissue culture plates containing 100 μl media/well. Cells were plated in media containing 10% serum. 6 h after plating, the media was replaced with media containing various concentrations of the test reagents and 10% serum. The plates were incubated for 72 h at 37°C. At the 72-h time point, cells were trypsinized and resuspended in PBS at 0.5–106 cells/ml. 1 μl from a solution of acridine orange (100 μg/ml) and ethidium bromide (100 μg/ml) was added to a 25-μl cell suspension, incubated for 2 min at room temperature, and examined under 40× magnification using a Zeiss Fluorescence microscope.

Test reagents were added to media alone in order to provide a blank.

To measure lactate dehydrogenase (LDH) release from cells, the colorimetric Cytotox 96-LDH-Release Assay™ (Promega Corp., Madison, WI) was performed according to the instructions of the manufacturer.

**Figure 2. FACS analysis of α5β1 integrin expression on IMR-32 neuroblastoma cells and CHO cells.** α5β1 surface expression by three IMR-32 cell clones transfected with human α5 cDNA, IMR-32/α5β1 clone 4 (A), IMR-32/α5β1 clone 15 (B), and IMR-32/α5β1 clone 16 (C; solid line) is compared with a vector-transfected control line IMR32/c (A and B; dashed lines), and the parental cell line IMR-32/p (C; dashed line). (D) CHO cells transfected with the human α5 cDNA express α5β1 on their surface (solid line), whereas the vector-transfected control cells (CHO-B2/c) do not (dashed line). Cells were stained with a monoclonal antibody to the human α5 integrin subunit, followed by an FITC-labeled secondary antibody, and analyzed by FACS. (E) CHO cells transfected with the human αv cDNA express αvβ1 on their surface (solid line), whereas the vector-transfected control cells (CHO-B2/c) do not. The staining was with a monoclonal antibody to the human αv integrin subunit.
Results

The Integrin α5β1 Mediates Cell Adhesion to Nonfibrillar AB1-40

The RHD sequence in AB resembles the integrin recognition sequence RGID, and has been implicated in cell adhesion to AB via one or more of the β1 integrins (Ghiso et al., 1992; Sabo et al., 1995). We set out to determine which of the RGID-binding integrins bind to AB. A CHO cell line deficient in α5 integrin subunit expression (CHO-B2) was transfected with cDNA encoding human α5, αv, or vector alone, and was examined for its ability to adhere to a surface coated with Aβ1-40. Each of the integrin transfectants adhered to Aβ in a dose-dependent manner, but cells that received the vector alone attached to Aβ within the BSA background range (Fig. 1A). CHO-B2/α5β1+ cells adhered strongly to Aβ, and CHO-B2/αvβ1+ cells were moderately adhesive, whereas the control cells CHO-B2/c did not adhere above BSA background levels. FACS analysis indicated that CHO-B2/α5β1+ and CHO-B2/αvβ1+ cell transfectants were similar in their expression of the transfected integrin (Fig. 2, D and E). A control peptide in which the Aβ sequence is inverted (Aβ40-1) did not have adhesive activity with any of the cell types tested (Fig. 1B). In addition, integrin transfectants adhered only to soluble nonfibrillar Aβ1-40, and not to fibrillar Aβ1-40 (Fig. 1B). Plates were coated with equal amounts of soluble and fibrillar Aβ1-40 as measured by [125I]Aβ1-40. The α5β1-mediated cell adhesion to soluble Aβ1-40 was inhibitable by the integrin-binding peptide GRGDSP, and by a function-blocking anti-α5 integrin monoclonal antibody (P1D6; Fig. 1C), but not by the control peptide GRGESP or a monoclonal antibody to αv (Fig. 1C). The αvβ3 integrin, which also binds to RGD, does not mediate adhesion to Aβ because avβ3-expressing IMR-90 cells did not adhere to Aβ when the α5 and αvβ3 integrins were blocked with anti-α5 and anti-β1 monoclonal antibodies (data not shown).

We also tested the α5-negative human neuroblastoma cell line IMR-32 (Neill et al., 1994) for Aβ attachment with (IMR-32/α5β1+) and (IMR-32/α5β1−) and without (IMR-32/c) α5 transfection (Fig. 2A). Three separate clones were obtained that expressed human α5β1 on their surface as detected by FACS analysis (Fig. 2, A–C). Each α5β1-expressing clone adhered to coated Aβ1-40 in a dose-dependent manner (Fig. 3A), and cell adhesion was inhibitable by an anti-α5 antibody (data not shown). The control-transfected IMR-32 cells (Fig. 2, A–C) attached poorly to this substrate (Fig. 3A). Both the transfected and control cells attached well to vitronectin (data not shown), whereas the control peptide Aβ40-1 and fibrillar Aβ1-40 did not promote adhesion above BSA background levels for any of the IMR-32 cell lines (Fig. 3B).

α5β1 Reduces the Formation of an Insoluble Aβ Fibrillar Extracellular Matrix

An increase of insoluble Aβ fibrillar matrix is one hallmark of AD (Glennan and Wong, 1984; Masters et al., 1985). As shown above, the α5β1 integrin bound to coated Aβ with the highest avidity among the integrins we tested. Therefore, we asked whether α5β1 would affect the formation of an Aβ fibrillar matrix. Exogenous Aβ1-40

Figure 3. Adhesion of IMR-32 cells to coated Aβ. (A) Adhesion of IMR-32 cells transfected with the human α5 subunit IMR-32/α5β1 and vector-transfected IMR-32/c cells to Aβ1-40 was measured as described in the legend for Fig. 1. (B) IMR-32 cell transfectants and control IMR-32/c and IMR-32/p cells were plated on Aβ1-40 coated in its soluble or fibrillar form or on the control peptide Aβ40-1, and cell adhesion was measured as described in the legend for Fig. 1. Values in A and B represent the mean ± SD, n = 9.
added to cell cultures formed a matrix around the cells that was detectable by immunostaining with anti-\( \alpha \)b antibodies. There was a substantial decrease in the formation of matrix from added A\( \beta \) in cultures of the \( \alpha \)5\( \beta \)1-expressing IMR-32 cell lines compared with the control lines (Fig. 4, A–D). Moreover, the matrix in the \( \alpha \)5\( \beta \)1+ cell cultures appeared to be cell-associated, whereas in the \( \alpha \)5\( \beta \)1− cell cultures it appeared to be largely independent of the cells.

To study quantitatively the formation of the A\( \beta \) matrix, the various IMR-32 lines were incubated with \( ^{125} \)I-labeled A\( \beta \) for 72 h, and the amount of radiolabeled A\( \beta \) that had become soluble in detergent was measured. The IMR-32 clones expressing \( \alpha \)5\( \beta \)1 deposited fivefold less insoluble A\( \beta \) radioactivity than the control cells. Moreover, the P1D6 anti-\( \alpha \)5 antibody returned A\( \beta \) matrix formation in the \( \alpha \)5\( \beta \)1-expressing IMR-32 cultures to the level in the parental control cells (Fig. 5 A). A control antibody had no effect. CHO cells expressing \( \alpha \)5\( \beta \)1 also had less A\( \beta \) matrix than their control-transfected counterpart cells as judged from the insolubility of \( ^{125} \)I\( \alpha \)B; the difference was fourfold (Fig. 5 B). Adding the anti-\( \alpha \)5 antibody canceled the \( \alpha \)5\( \beta \)1 effect, but a control antibody did not. The insolubility of A\( \beta \) remained the same in the CHO control cell cultures regardless of the antibody added. These results indicate that cell expression of \( \alpha \)5\( \beta \)1 reduces A\( \beta \) matrix deposition threefold relative to the control cells. Because iodinated A\( \beta \) forms fibrils less readily than unlabeled A\( \beta \)1-40 (Bush et al., 1994), it was not possible to use the \( ^{125} \)I\( \alpha \)B to quantitate the proportion of the added A\( \beta \)1-40 that becomes insolubilized.

**Soluble A\( \beta \)1-40 is Taken Up By Cells and Partially Degraded Via an \( \alpha \)5\( \beta \)1-Mediated Pathway**

Possible reasons for the \( \alpha \)5\( \beta \)1-mediated reduction of A\( \beta \) matrix include internalization of soluble A\( \beta \)1-40, degradation of the peptide, or both. Neuronal cells have been shown to internalize A\( \beta \), but the mechanism for this internalization is only incompletely known (Ida et al., 1996, Hammad et al., 1997). To investigate the possibility that binding \( \alpha \)5\( \beta \)1 to soluble A\( \beta \) initiates cellular uptake of A\( \beta \), we examined the processing of \( ^{125} \)I-labeled A\( \beta \)1-40 by \( \alpha \)5\( \beta \)1+ and \( \alpha \)5\( \beta \)1− cells. Initially, CHO-B2/c control cells and transfectants were incubated for 1 h with \( ^{125} \)I\( \alpha \)B-40, and were then examined for cell-associated radioactivity. The \( \alpha \)5\( \beta \)1-expressing CHO-B2 cells contained twofold more radioactivity at 1 and 12 h than the control CHO-B2/c cells (Fig. 6 A).

Cell cultures were then incubated with \( ^{125} \)I-labeled A\( \beta \) over a 72-h period to determine whether the \( ^{125} \)I\( \alpha \)B taken up by the cells was degraded. \( \alpha \)5\( \beta \)1-expressing IMR-32 cells contained twofold more radioactivity after the 72-h in-
culture medium. The release of radioactivity into cell culture media was monitored over a 72-h period that followed incubation with 125I-labeled soluble Aβ1-40, which was incubated with cultures of two control lines and three α5β1-expressing lines at 37°C for 72 h in the presence of either the monoclonal antibody to the human α5 integrin subunit (P1D6) or control IgG. The total amount of 125I-labeled Aβ1-40 associated with an SDS-soluble matrix was determined as described in Materials and Methods. The reduction of Aβ1-40 matrix deposition in the α5β1-expressing cultures was reversed by an anti-α5 integrin monoclonal antibody (P1D6), but not by the control IgG. (B) CHO-B2/α5β1 cells show reduced deposition of [125I] to Aβ matrix relative to control CHO cells. The experimental procedure was the same as in A. The P1D6 antibody reversed the α5β1-dependent matrix reduction, whereas the control mouse IgG had no effect. Experiments in A and B were repeated at least four times, and representative results are shown. Values represent the mean ± SD; n = 3.

Figure 5. Quantitation of Aβ matrix deposition in cultures of IMR-32 cells and their α5β1-expressing clones. (A) 125I-labeled soluble Aβ1-40 was incubated with cultures of two control lines and three α5β1-expressing lines at 37°C for 72 h in the presence of either the monoclonal antibody to the human α5 integrin subunit (P1D6) or control IgG. The total amount of 125I-labeled Aβ1-40 associated with an SDS-soluble matrix was determined as described in Materials and Methods. The reduction of Aβ1-40 matrix deposition in the α5β1-expressing cultures was reversed by an anti-α5 integrin monoclonal antibody (P1D6), but not by the control IgG. (B) CHO-B2/α5β1 cells show reduced deposition of [125I] to Aβ matrix relative to control CHO cells. The experimental procedure was the same as in A. The P1D6 antibody reversed the α5β1-dependent matrix reduction, whereas the control mouse IgG had no effect. Experiments in A and B were repeated at least four times, and representative results are shown. Values represent the mean ± SD; n = 3.

A

B

P1D6 antibody reversed the α5β1-dependent matrix reduction, whereas the control mouse IgG had no effect. Experiments in A and B were repeated at least four times, and representative results are shown. Values represent the mean ± SD; n = 3.

α5β1 Protects Cells Against Aβ Induced Apoptosis

Having established an α5β1-dependent mechanism for the inhibition of Aβ matrix deposition, we examined whether the reduction of the Aβ matrix would promote neuronal cell survival in cultures treated with Aβ. IMR-32 cell lines cultured with exogenous soluble Aβ1-40 underwent apoptosis in the absence of α5β1 (Fig. 7, A and B), but three α5β1-expressing lines did not (two are shown in Fig. 7, C and D). The control peptide Aβ40-1 caused no apoptosis in the control (Fig. 7, E and F) or α5β1-expressing cells (not shown). Analysis of acridine orange/ethidium bromide uptake revealed three times more apoptosis in the control cells than in the IMR-32 α5β1-expressers (Fig. 8).

We also assessed the Aβ effect by using the MTT assay, which measures cell viability by detecting the ability of a mitochondrial enzyme to reduce its substrate. Aβ-treated IMR-32 control cells lost their ability to reduce MTT in a manner that was dependent on the dose of Aβ, whereas Aβ had almost no effect on the α5β1-expressing cell lines (Fig. 9 A). The control peptide Aβ40-1 had no effect on MTT reduction in any of the cell types, even at the highest test concentration (Fig. 9 B). To examine further the cytotoxicity of Aβ1-40, we used an assay that measures the release of LDH upon cell lysis (Behl et al., 1994). A threefold increase in LDH levels relative to controls was seen in the α5β1- IMR-32 cells cultured in the presence of Aβ1-40, whereas Aβ1-40 had no effect on the LDH levels of the α5β1+ cells (Fig. 10 A). These results indicate that α5β1-mediated Aβ binding protects the IMR-32 cells from the cytotoxicity of aggregated Aβ, presumably by inhibiting its aggregation into fibrils. No apoptosis was caused by Aβ in any of the CHO cell lines, as examined by TUNEL staining, the MTT assay, and the LDH assay, indicating that these cells are resistant to the cytotoxic effects of an Aβ matrix.

We previously demonstrated that cell attachment through α5β1 protects CHO cells from apoptosis when cultured in a serum-free environment (Zhang et al., 1995). Therefore, we examined whether ligation of α5β1 to coated Aβ1-40
would protect α5β1-expressing CHO cells from apoptosis in serum-free cultures. CHO-B2/α5β1+ cells were plated on either fibronectin, vitronectin, or Aβ-coated dishes and examined for survival 96 h after serum withdrawal. CHO-B2/α5β1+ cells survived on Aβ and fibronectin, whereas cells plated on vitronectin underwent apoptosis (Fig. 10B). These results indicate that α5β1 can also protect cells from apoptosis by mediating cell attachment to coated Aβ.

Discussion

We report that the α5β1 integrin mediates cell adhesion to Aβ and promotes internalization and degradation of Aβ. This α5β1–Aβ interaction correlates with both an increase in the clearance of soluble Aβ, a reduction in the formation of an insoluble Aβ fibrillar matrix, and a decrease of the toxicity of Aβ to cells. This study provides one mechanism for regulating Aβ accumulation.

Our data, showing that Aβ binds to α5β1, and to a lesser extent αvβ1, is in agreement with previous reports that Aβ mediates cell attachment, and that the RHD sequence in it serves as an integrin-binding site (Ghiso et al., 1992; Sabo et al., 1995). The RHD sequence apparently functions as a mimic of the RGD sequence in fibronectin, the matrix ligand of α5β1 (Ruoslahti, 1996a), because a short peptide containing the RGD sequence inhibits Aβ binding. α5β1 binds only to nonfibrillar Aβ, since we did not see any detectable cell adhesion to aggregated fibrillar Aβ. Therefore, other receptors presumably mediate cellular interactions with fibrillar Aβ, and are responsible for the cytotoxic effects of this form of Aβ. The α5β1 integrin is one of the most discriminating of the RGD-directed integrins in regard to its ligand specificity (Ruoslahti, 1996b). In addition to its main ligand fibronectin, the α5β1 integrin has only been shown to bind to the bacterial protein invasin (Watari et al., 1996) and the insulin-like growth factor binding protein IGFBP-X (Jones et al.,...
Our results add Aβ among its ligands. The binding site for α5β1 seems to be available only in Aβ, not in its precursor protein (APP; B. Bossy, M.L. Matter, and E. Ruoslahti, unpublished results).

The α5β1 integrin may play a role in the rapid clearance of Aβ that occurs in the normal brain (Ghersi-Egea et al., 1996). We show that expression of the α5β1 integrin is associated with increased cellular uptake and degradation and decreased matrix deposition of Aβ in cell cultures. Moreover, reversal of this effect with a function-blocking anti-α5 antibody established a causal link between α5β1 activity and increased clearance of Aβ. Although more complex explanations of this effect are possible, the binding of Aβ to α5β1 shown here suggests that Aβ binds to α5β1 at the cell surface, and is subsequently internalized into a cellular compartment where it is degraded. This hypothesis is in agreement with previous results showing that a neuronal cell line internalizes Aβ from culture medium in a manner that is dependent on the NH2 terminus of Aβ where the RHD sequence resides (Ida et al., 1996). The lipoprotein Apo J can also reduce the formation of fibrillar Aβ by causing it to be internalized and degraded (Hammad et al., 1997). Thus, it is likely that more than one mechanism plays a role in the regulation of Aβ accumulation in vivo. Clearly, a transgenic animal expressing the amyloid precursor protein with a mutated RHD sequence would be of great interest in testing the contribution of the RHD sequence and integrin-binding to the metabolism of Aβ.

The α5β1 integrin circulates through the endocytic cycle (Bretscher, 1989; Bretscher, 1992). Inhibiting exocytosis with primaquin causes accumulation of internalized Aβ in an intracellular pool that returns to the cell surface over time. Recent studies have shown that internalization of fibrillar Aβ promotes accumulation of stable fibrillar Aβ in the late endosome/secondary lysosome compartment, whereas internalization of soluble Aβ leads to degradation of the peptide in the same compartment (Knauer et al., 1992; Koo and Squazzo, 1994; Yang et al., 1995). This result is in agreement with our data, showing that soluble Aβ is internalized through an α5β1 integrin-mediated pathway, and is at least partially degraded, presumably within endosomes. Thus, clearance of soluble Aβ can be mediated by the α5β1 integrin, presumably through the receptor-
mediated endocytosis pathway that normally internalizes this integrin.

α5β1 may play a protective role in the brain by suppressing Aβ cytotoxicity. We provide evidence for two separate mechanisms that could be responsible for such a protective effect. First, we show that α5β1-mediated adhe-
sion to nonfibrillar Aβ protects cells from apoptosis in cell culture. Upregulation of Bel-2 (Zhang et al., 1995) and activation of the MAPK pathway (Wary et al., 1997) may be responsible for this pathway. The second and potentially more important mechanism is suggested by our demonstration that α5β1 suppresses the apoptotic effects of Aβ by reducing production of toxic Aβ matrix.

The α5β1 integrin and αvβ1 are present in the adult central nervous system (Grooms et al., 1993). Immunostaining for α5β1 shows that it is expressed in the vasculature, cortex, and hippocampus of adult rat brain (Bahr et al., 1991; Pagani et al., 1992; Tawil et al., 1994; for review see Sargent Jones, 1996). Moreover, primary hippocampal neurons express α5β1 (Yamazaki et al., 1997), Soluble Aβ1-40 is present in vivo (Seubert et al., 1992), and is rapidly cleared when injected into normal rats (Ghersi-Egea et al., 1996). Our results suggest that α5β1 may mediate the clearance of Aβ, and that α5β1 may play a significant role in protecting the brain from the Aβ-initiated pathology that in its extreme form causes AD.

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