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.

Integrin-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 Bcl-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-asp (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*a*).

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

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1. *Abbreviations used in this paper*: Aβ, amyloid β peptide; AD, Alzheimer's disease, LDH, lactate dehydrogenase; RHD, arg-his-asp.

this interaction promotes clearance of Aβ by cultured cells, reducing the formation of an insoluble Aβ fibrillar matrix and counteracting 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 adhesive activity (lots zn571 and wm365), while lot zn327 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, Switerland). 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. Fibronectin 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/αvβ1$^+$, 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 (L230), 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 fibronectin 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 (2×10^5 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 [^{125}I]Aβ1-40 or preaggregated [^{125}I]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, pH 7.4, for 30 min at room temperature. The cultures were then blocked with 1% BSA/PBS and stained with a polyclonal 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 [^{125}I]Aβ

^{125}I-labeled Aβ1-40 peptide was purchased as a lyophilized powder (25 μCi) from Nycomed 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 ^{125}I-labeled Aβ1-40 peptide was 2×10^6 μCi/mmol.

Insolubilization of Aβ was analyzed using ^{125}I-labeled Aβ1-40 peptide as described previously for fibronectin matrix assembly (McKeown-Longo and Mosher, 1985; Morla and Ruoslahti, 1992). Cells were plated at 10^5 cells/ml (IMR variants) or 0.5×10^5 cells/ml (CHO variants) into 24-well tissue culture plates in media containing 10% serum. Media was replaced 6 h after plating with media containing [^{125}I]Aβ1-40 and 10% serum. Cells were cultured for 72 h at 37°C. The media was then removed, the wells were washed three times with PBS, and 5× SDS sample buffer (0.5M Tris pH 6.8, glycerol, 10% SDS, 0.5% bromophenol blue) was used to solubilize the [^{125}I]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. ^{125}I-labeled 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 [^{125}I]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 [^{125}I]Aβ1-40 (2 ng/ml). The cells were incubated for 1 h with [^{125}I]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, 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 [^{125}I]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 ^{125}I-Labeled Aβ1-40

Subconfluent cells were detached with trypsin, washed once with media, and plated at 10^5 cells/ml in 24-well plates. 6 h after plating, media was replaced with 2 ng/ml of [^{125}I]Aβ1-40 in serum-containing media and incubated for 1 h at 37°C. The radiolabeled media was removed, and cells were

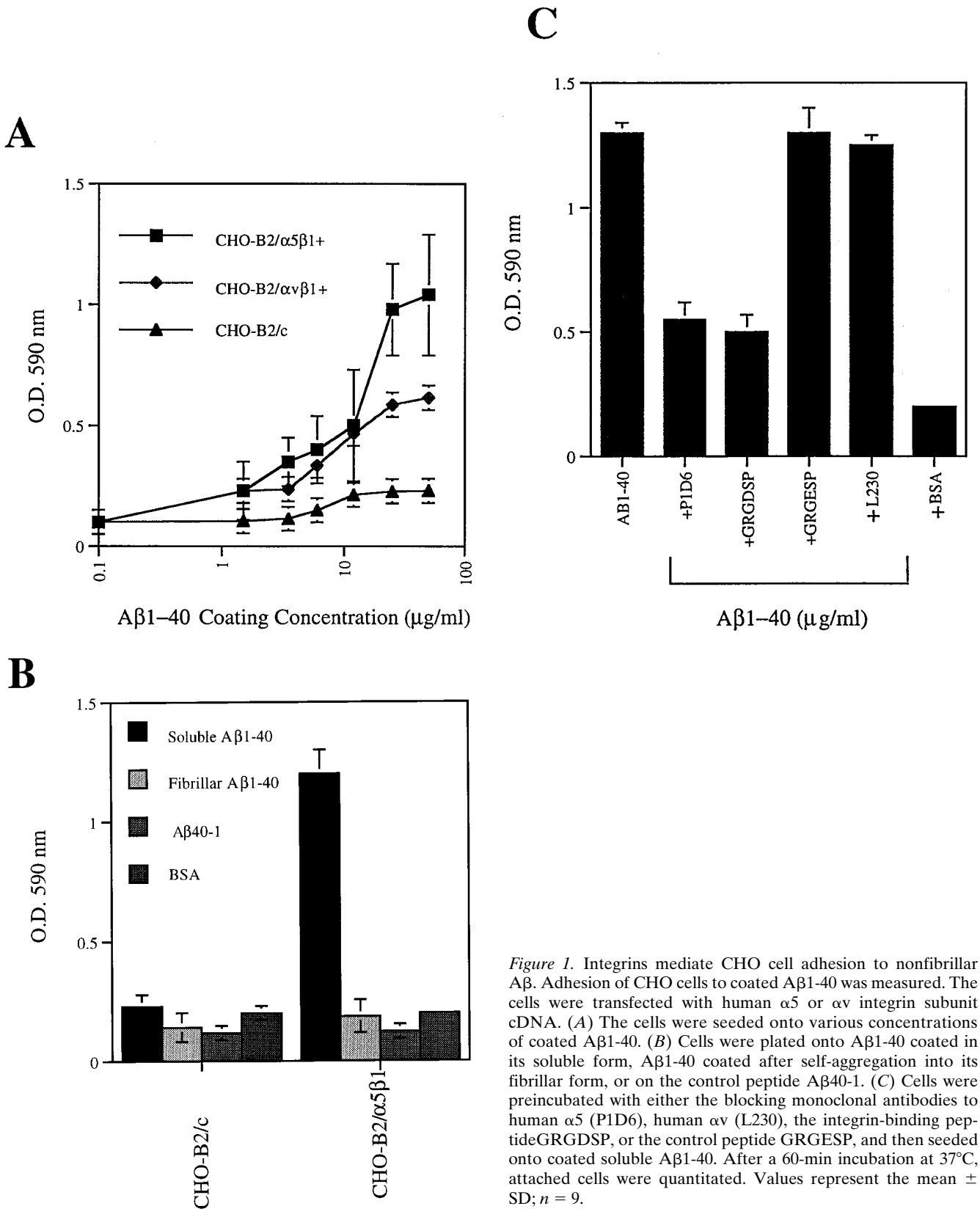

Figure 1. Integrins mediate CHO cell adhesion to nonfibrillar Aβ. Adhesion of CHO cells to coated Aβ1-40 was measured. The cells were transfected with human α5 or αv integrin subunit cDNA. (*A*) The cells were seeded onto various concentrations of coated Aβ1-40. (*B*) Cells were plated onto Aβ1-40 coated 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 GRGESP, 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 [^{125}I] 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 acridine 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 0.5×10^6 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.

Cells cultured in microtiter wells were pulsed with 25 μl of a 2.5 mg/ml MTT stock in PBS and incubated for 4 h. Then 100 μl of a solution containing 10% SDS, 0.01 N HCl was added, and the plates were incubated overnight (Tada et al., 1986). Absorption was read on a Vmax Microplate Reader™ (Molecular Devices Corp., Sunnyvale, CA) using a reference wavelenth of 650 nm and a test wavelength of 590 nm. 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.

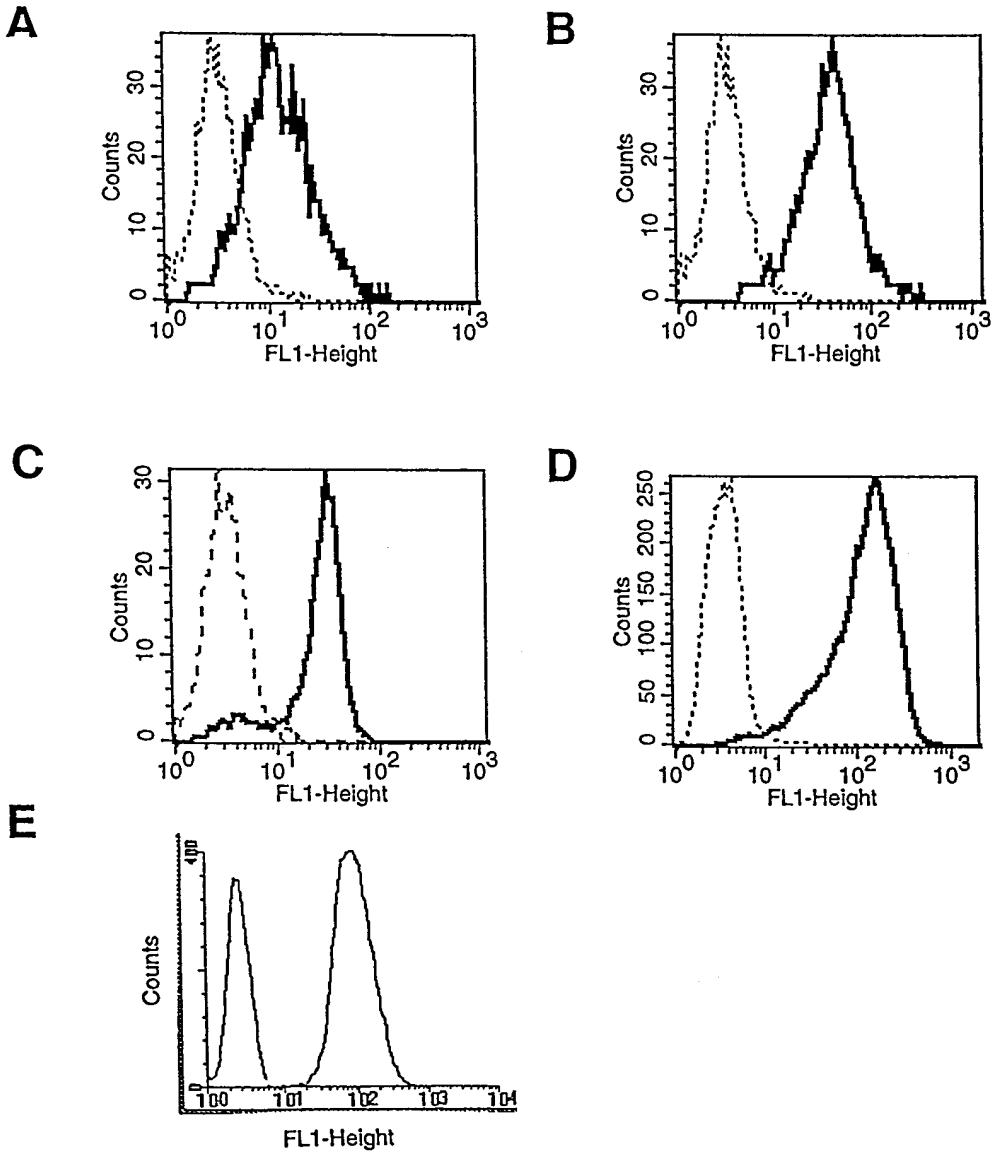

Figure 2. FACS analysis of α5β1 integrin expression on IMR-32 neuroblastoma cells and CHO cells. α5β1 surface expression by three IMR-32 cell clones transfected with human α5 cDNA. IMR-32/α5β1 clone 4 (*A*), IMR-32/α5β1 clone 15 (*B*), and IMR-32/α5β1 clone 16 (*C*; *solid lines*) is compared with a 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 Aβ1-40

The RHD sequence in Aβ resembles the integrin recognition sequence RGD, and has been implicated in cell adhesion to Aβ via one or more of the β1 integrins (Ghiso et al., 1992; Sabo et al., 1995). We set out to determine which of the RGD-binding integrins bind to Aβ. 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. 1 *A*). 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. 1 *B*). In addition, integrin transfectants adhered only to soluble nonfibrillar Aβ1-40, and not to fibrillar Aβ1-40 (Fig. 1 *B*). Plates were coated with equal amounts of soluble and fibrillar Aβ1-40 as measured by [^{125}I]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. 1 *C*), but not by the control peptide GRGESP or a monoclonal antibody to αv (Fig. 1 *C*). The αvβ3 integrin, which also binds to RGD, does not mediate adhesion to Aβ because αvβ3-expressing IMR-90 cells did not adhere to Aβ when the α5β1 and αvβ1 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 without (IMR-32/c) α5 transfection (Fig. 2 *A*). 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. 3 *A*), 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. 3 *A*). 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. 3 *B*).

α5β1 Reduces the Formation of an Insoluble Aβ Fibrillar Extracellular Matrix

An increase of insoluble Aβ fibrillar matrix is one hallmark of AD (Glenner 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

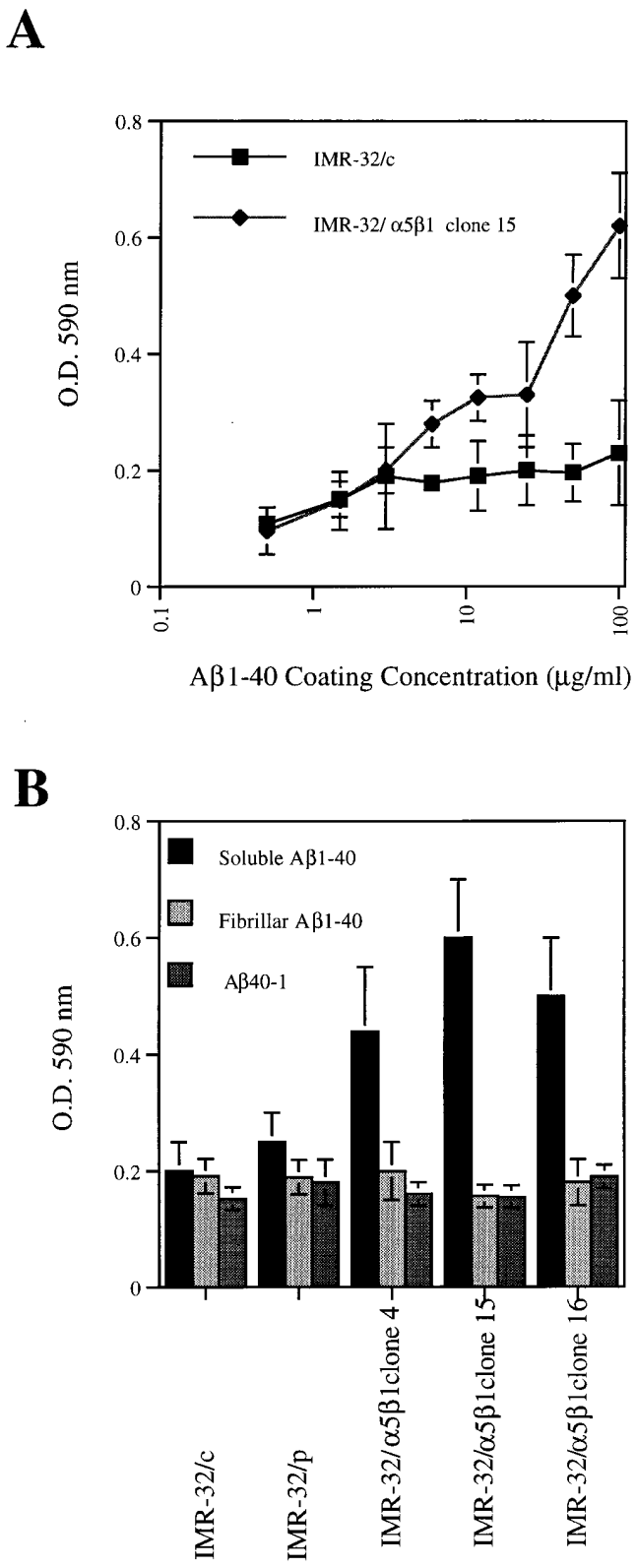

Figure 3. Adhesion of IMR-32 cells to coated Aβ. (*A*) Adhesion of IMR-32 cells transfected with the human α5 subunit IMR-32/α5β1 and vector-transfected IMR-32/c cells to Aβ1-40 was measured as described in the legend for Fig. 1. (*B*) IMR-32 cell transfectants and control IMR-32/c and IMR-32/p cells 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; $n = 9$.

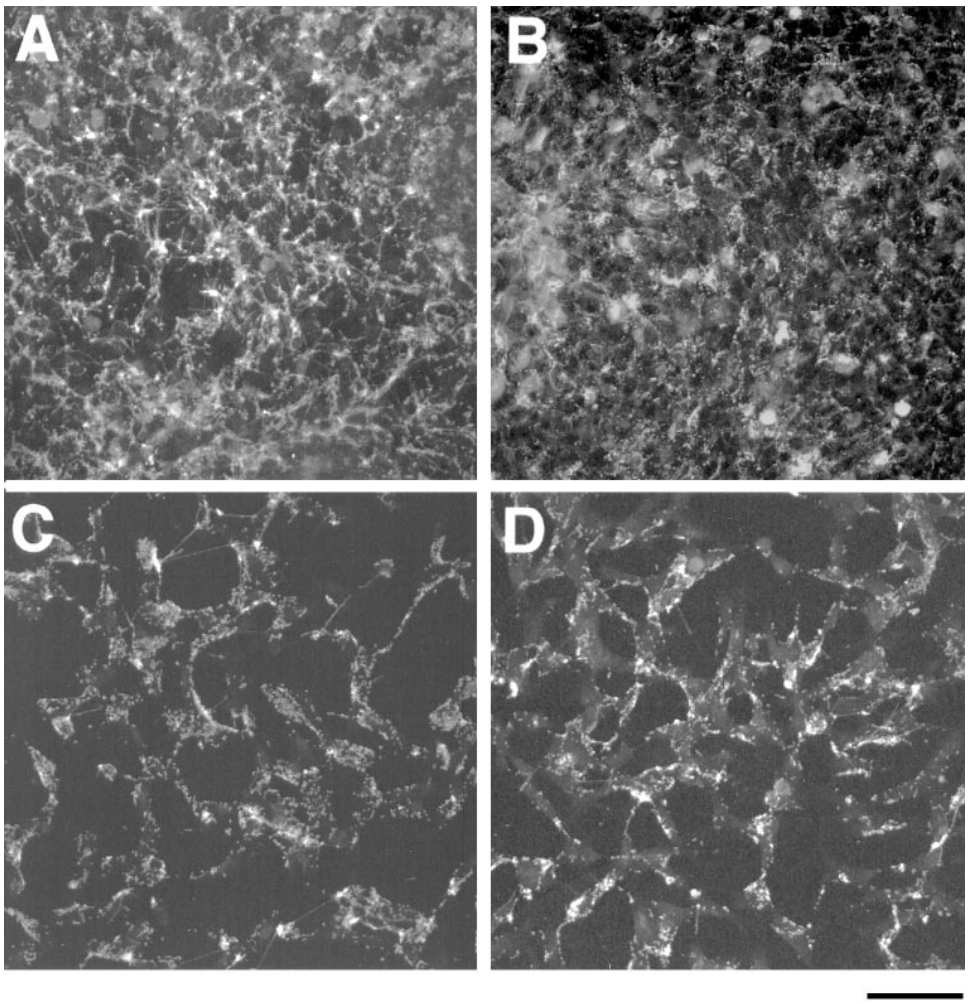

Figure 4. Immunofluorescent detection of reduced Aβ matrix in cultures expressing the α5β1 integrin. Deposition of Aβ1-40 matrix from soluble Aβ added to the culture media of IMR-32 control cells IMR-32/c (*A*) and IMR-32/p (*B*), and of α5β1 expressing IMR-32 clones 4 (*C*) and 15 (*D*) was examined. The Aβ matrix was detected by a polyclonal anti-Aβ1-40 antibody followed by a rhodamine-labeled secondary antibody. The experiment was repeated at least three times for each cell line, and representative results are shown. Bar, 50 μm.

added to cell cultures formed a matrix around the cells that was detectable by immunostaining with anti-Aβ antibodies. There was a substantial decrease in the formation of matrix from added Aβ in cultures of the α5β1-expressing IMR-32 cell lines compared with the control lines (Fig. 4, *A–D*). Moreover, the matrix in the $\alpha5\beta1^+$ cell cultures appeared to be cell-associated, whereas in the $\alpha5\beta1^-$ cell cultures it appeared to be largely independent of the cells.

To study quantitatively the formation of the Aβ matrix, the various IMR-32 lines were incubated with ^{125}I-labeled Aβ for 72 h, and the amount of radiolabeled Aβ that had become soluble in detergent was measured. The IMR-32 clones expressing α5β1 deposited fivefold less insoluble Aβ radioactivity than the control cells. Moreover, the P1D6 anti-α5 antibody returned Aβ matrix formation in the α5β1-expressing IMR-32 cultures to the level in the parental control cells (Fig. 5 *A*). A control antibody had no effect. CHO cells expressing α5β1 also had less Aβ matrix than their control-transfected counterpart cells as judged from the insolubility of [^{125}I]Aβ; the difference was fourfold (Fig. 5 *B*). Adding the anti-α5 antibody canceled the α5β1 effect, but a control antibody did not. The insolubility of Aβ remained the same in the CHO control cell cultures regardless of the antibody added. These results indicate that cell expression of α5β1 reduces Aβ matrix deposition threefold relative to the control cells. Because iodinated Aβ forms fibrils less readily than unlabeled Aβ1-40 (Bush et al., 1994), it was not possible to use the [^{125}I]Aβ to quantitate the proportion of the added Aβ1-40 that becomes insolubilized.

Soluble Aβ1-40 is Taken Up By Cells and Partially Degraded Via an α5β1-Mediated Pathway

Possible reasons for the α5β1-mediated reduction of Aβ matrix include internalization of soluble Aβ1-40, degradation of the peptide, or both. Neuronal cells have been shown to internalize Aβ, but the mechanism for this internalization is only incompletely known (Ida et al., 1996, Hammad et al., 1997). To investigate the possibility that binding α5β1 to soluble Aβ initiates cellular uptake of Aβ, we examined the processing of ^{125}I-labeled Aβ1-40 by $\alpha5\beta1^+$ and $\alpha5\beta1^-$ cells. Initially, CHO-B2/c control cells and transfectants were incubated for 1 h with [^{125}I]Aβ1-40, and were then examined for cell-associated radioactivity. The α5β1-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 Aβ over a 72-h period to determine whether the [^{125}I]Aβ taken up by the cells was degraded. α5β1-expressing IMR-32 cells contained twofold more radioactivity after the 72-h in-

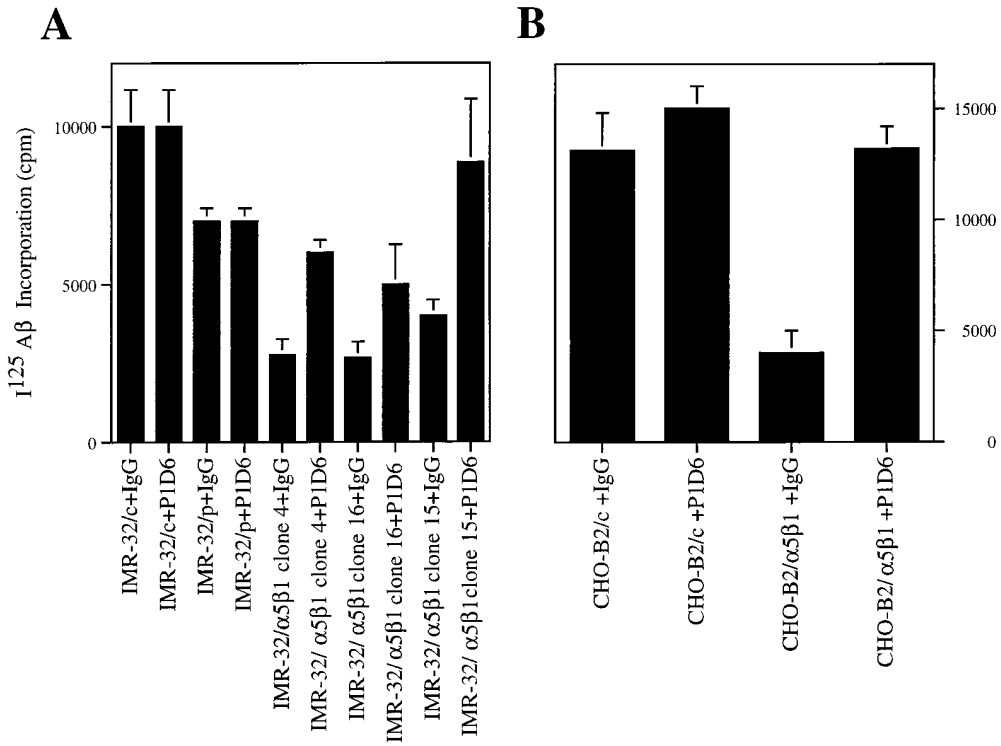

Figure 5. Quantitation of Aβ matrix deposition in cultures of IMR-32 cells and their α5β1-expressing clones. (*A*) ^{125}I-labeled soluble Aβ1-40 was incubated with cultures of two control lines and three α5β1-expressing lines at 37°C for 72 h in the presence of either the monoclonal antibody to the human α5 integrin subunit (P1D6) or control IgG. The total amount of ^{125}I-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 [^{125}I] 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; $n = 3$.

cubation than α5β1-negative IMR-32 cells (Fig. 6 *B*). Part of the radioactivity was soluble in TCA, indicating that Aβ had been degraded. CHO cells internalized and degraded soluble Aβ in a similar manner, with α5β1-expressing cells containing eightfold more TCA-soluble radioactivity than α5β1-negative cells (Fig. 6 *C*). The CHO cells expressing α5β1 bound 10% of the added Aβ, whereas the control cells bound only 0.4%. Moreover, 90% of the cell-associated Aβ was degraded in the CHO-α5β1 expressers. The higher expression levels of α5β1 on the CHO transfectants (Fig. 2, *D* and *E*) may explain why these cells bound and internalized more radiolabeled Aβ than the IMR-32 transfectants.

We next examined whether Aβ was released into the culture medium. The release of radioactivity into cell culture media was monitored over a 72-h period that followed a 1-h incubation with ^{125}I-labeled Aβ1-40. The media of α5β1-expressing IMR-32 and CHO cells contained twofold more radioactivity than the corresponding control cell media (Fig. 6, *D* and *E*). These results point to an α5β1-dependent pathway that internalizes and degrades Aβ.

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 $\alpha5\beta1^-$ IMR-32 cells cultured in the presence of Aβ1-40, whereas Aβ1-40 had no effect on the LDH levels of the $\alpha5\beta1^+$ 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

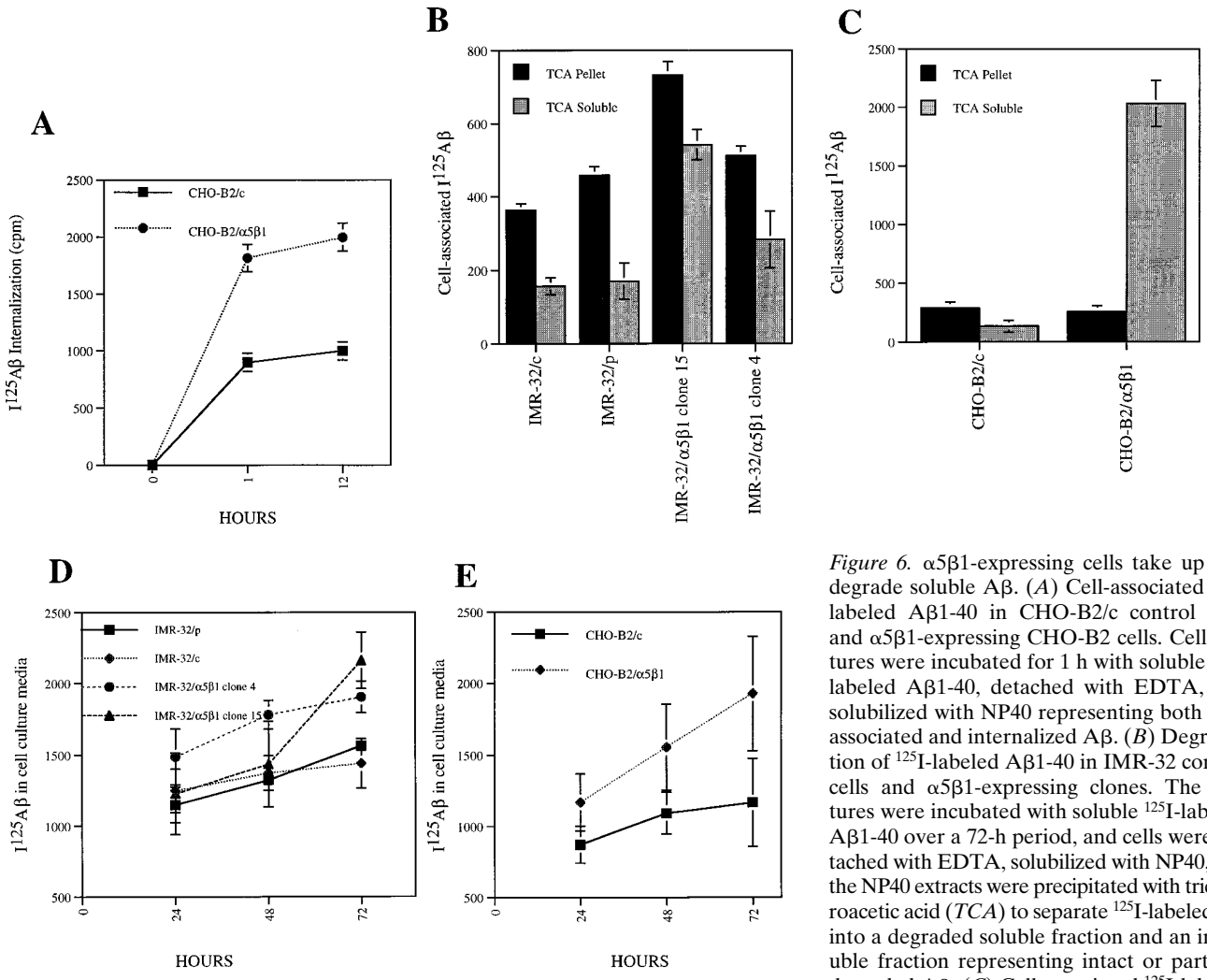

Figure 6. α5β1-expressing cells take up and degrade soluble Aβ. (*A*) Cell-associated ^{125}I-labeled Aβ1-40 in CHO-B2/c control cells and α5β1-expressing CHO-B2 cells. Cell cultures were incubated for 1 h with soluble ^{125}I-labeled Aβ1-40, detached with EDTA, and solubilized with NP40 representing both cell-associated and internalized Aβ. (*B*) Degradation of ^{125}I-labeled Aβ1-40 in IMR-32 control cells and α5β1-expressing clones. The cultures were incubated with soluble ^{125}I-labeled Aβ1-40 over a 72-h period, and cells were detached with EDTA, solubilized with NP40, and the NP40 extracts were precipitated with trichloroacetic acid (*TCA*) to separate ^{125}I-labeled Aβ into a degraded soluble fraction and an insoluble fraction representing intact or partially degraded Aβ. (*C*) Cell-associated ^{125}I-labeled Aβ1-40 in CHO control cells (CHO-B2/c) and α5β1-expressing clones (CHO-B2/α5β1). (*D* and *E*) Release of [^{125}I] from ^{125}I-labeled Aβ1-40 into cell culture medium. ^{125}I-labeled soluble Aβ1-40 was added to the culture media of various IMR-32 (*D*) or CHO (*E*) cell lines and incubated for 1 h. After that, the media was removed, the cell layer was washed, and new media was added. The release of radioactivity into the media was monitored over a 72-h period. Experiments in *A–E* were repeated at least three times, and representative results are shown. Values in *A–E* represent the mean ± SD; $n = 3$.

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. 10 *B*). 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, 1996*a*), 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 with regard to its ligand specificity (Ruoslahti, 1996*b*). 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.,

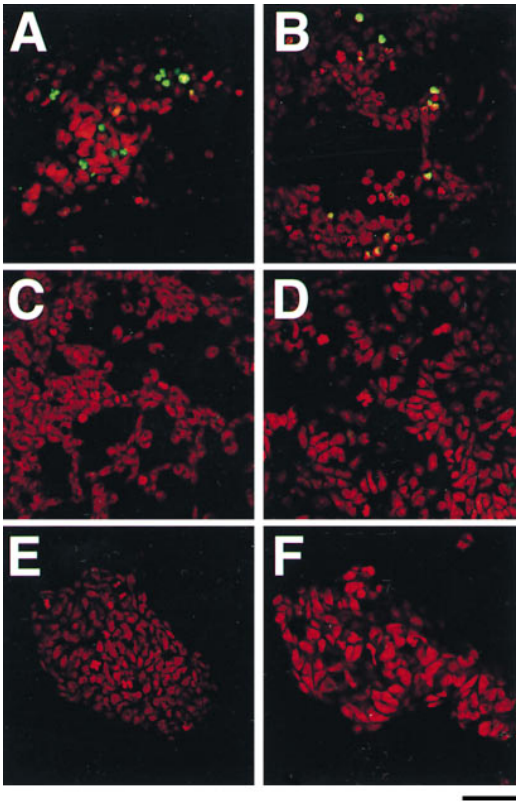

Figure 7. Effect of Aβ matrix on apoptosis. Expression of α5β1 protects IMR-32 neuroblastoma cells from apoptosis induced by Aβ matrix formation. Cell cultures were incubated for 72 h with Aβ1-40 to allow aggregation of Aβ into a matrix, and the cultures were then examined for evidence of cytotoxicity by using the TUNEL assay. A percentage of IMR-32/c (*A*) and IMR-32/p (*B*) cells underwent apoptosis (*green*). The viable cells (*red*) are counterstained with propidium iodide. No apoptotic cells were seen in IMR-32/α5β1 transfectant clones 4 (*C*) and 15 (*D*) under the same conditions. (*E–F*) The IMR-32/c and IMR-32/p control cells remained viable when incubated for 72 h with the inactive control peptide Aβ40-1. These experiments were repeated at least three times for each cell line, and representative results are shown. Bar, 50 μm.

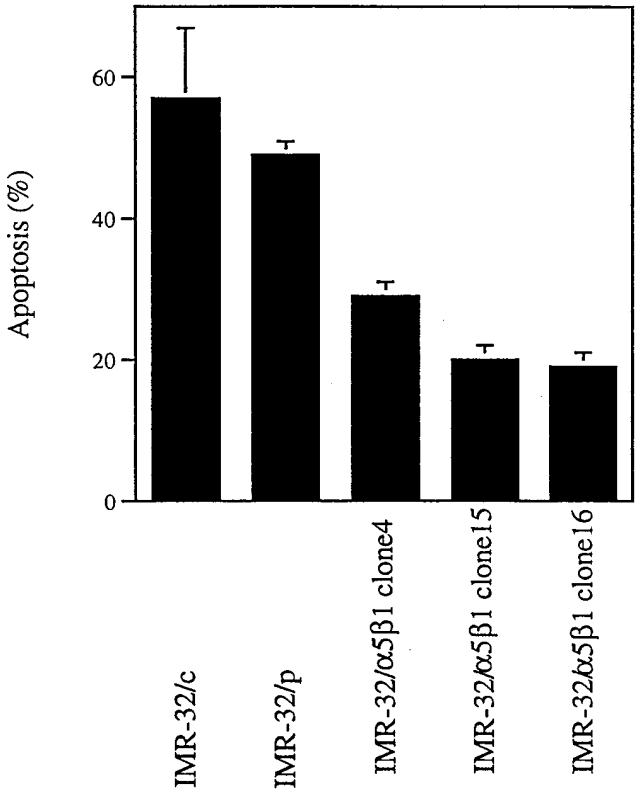

Figure 8. Effect of Aβ matrix on apoptosis. Expression of α5β1 protects IMR-32 neuroblastoma cells from apoptosis induced by Aβ matrix formation. Cell cultures were incubated for 72 h with Aβ1-40 to allow aggregation of Aβ into a matrix, and the cultures were then examined for evidence of apoptosis by measuring the ability of cells to take up acridine orange/ethidium bromide. A higher percentage of $\alpha5\beta1^-$IMR-32/c and IMR-32/p cells than $\alpha5\beta1^+$ transfectants underwent apoptosis. Values represent the mean ± SD; $n = 9$.

1993). 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 NH_2 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 α5β1 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-

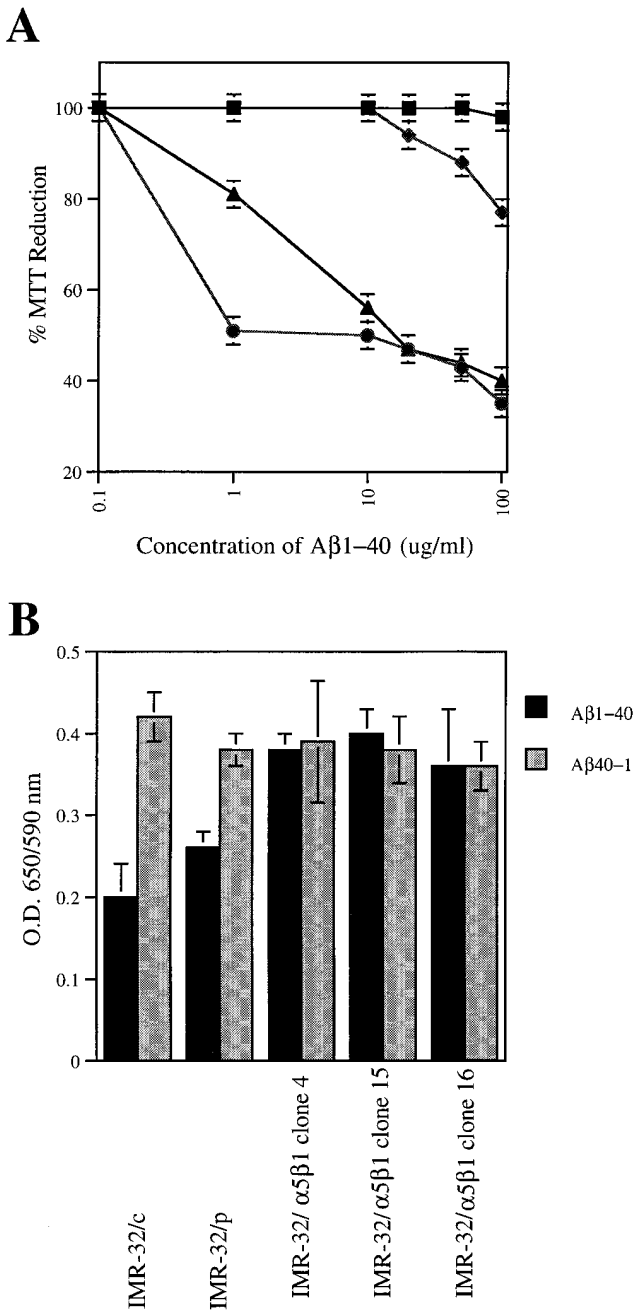

Figure 9. Effect of Aβ1-40 on cell viability. Cell survival in cultures containing aggregated Aβ. Cell cultures were incubated for 72 h with Aβ1-40 to allow aggregation of Aβ into a matrix, and the cultures were then examined for their ability to reduce MTT. (*A*) The control cells—IMR-32/c (*circles*) and IMR-32/p (*triangles*)—lost the ability to reduce MTT, whereas the IMR-32/α5β1 clones 4 (*diamonds*) and 15 (*squares*) were essentially resistant under the same conditions. Values represent the mean ± SD; n = 9. (*B*) The control peptide Aβ40-1 did not affect the ability of any cell type to reduce MTT.

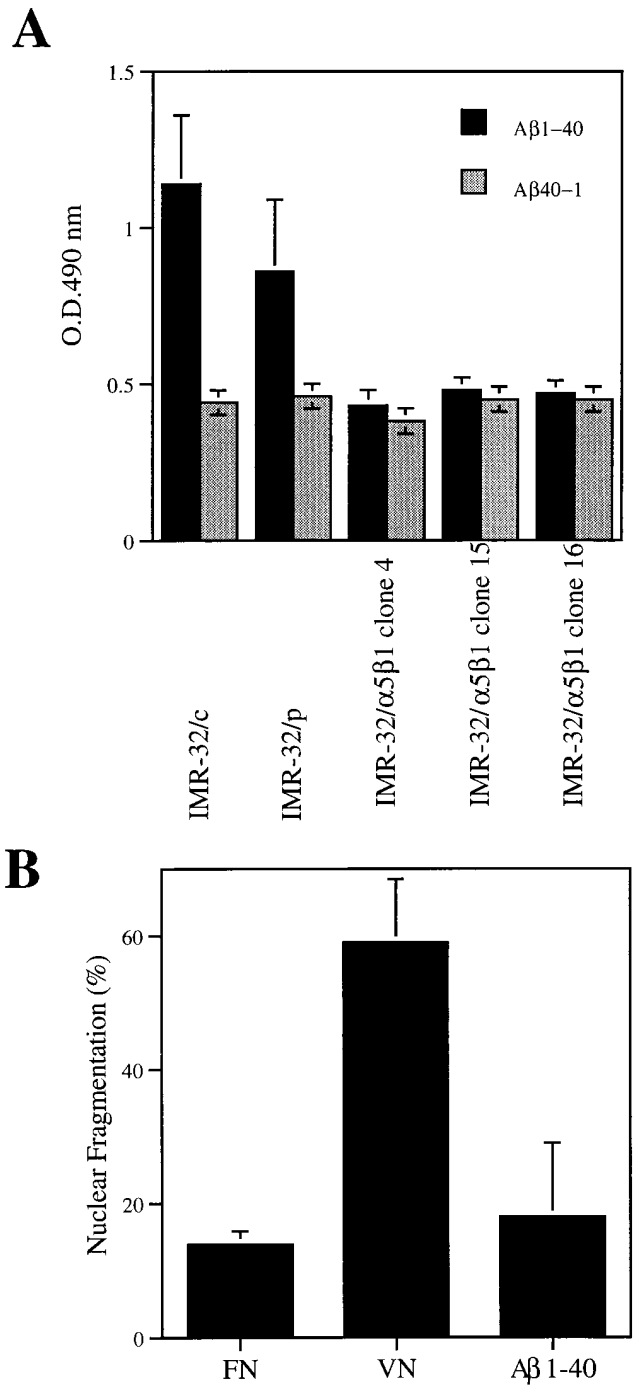

Figure 10. Cell survival in cultures containing aggregated Aβ. Cell cultures were incubated for 72 h with Aβ1-40 to allow aggregation of Aβ into a matrix, and the LDH levels within the cultures were analyzed. (*A*) The α5β1$^-$ cells IMR-32/c and IMR-32/p showed increased LDH levels, whereas the IMR-32/α5β1 clones 4, 15, and 16 maintained LDH levels similar to those seen in the presence of the Aβ40-1 control peptide. Values represent the mean ± SD; n = 9. (*B*) Cell survival on Aβ coated from freshly made solution onto a plastic surface. Cells were seeded onto Aβ1-40, fibronectin, and vitronectin coated onto microtiter wells, cultured in serum-free conditions, and examined by DAPI staining after 96 h. The α5β1 transfectants remained viable on Aβ1-40 and fibronectin, but not on vitronectin.

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 Bcl-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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