Imaging Amyloid Plaque in Alzheimer's Disease Brain with a Biotinylated Aβ Peptide Radiopharmaceutical Conjugated to an IgG–Avidin Fusion Protein

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Imaging Amyloid Plaque in Alzheimer's Disease Brain with a Biotinylated Aβ Peptide Radiopharmaceutical Conjugated to an IgG-Avidin Fusion Protein

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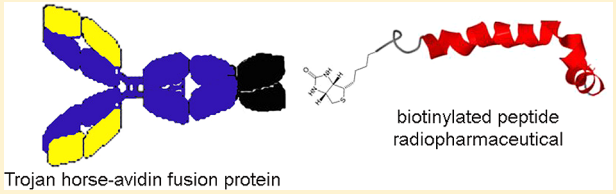
ABSTRACT: The Aβ amyloid peptide of Alzheimer's disease (AD) is a potentially large molecule radiopharmaceutical for imaging the brain amyloid burden, should the peptide be made transportable across the blood-brain barrier (BBB). Peptides can be made BBB-penetrating with the combined use of Trojan horse and avidin−biotin technologies. The peptide is monobiotinylated and attached to a fusion protein of avidin (AV) and a genetically engineered monoclonal antibody (mAb) against the human insulin receptor (HIR). The fusion protein is designated HIRMAb-AV, and is produced by stably transfected mammalian host cells grown in biotin free medium. The HIRMAb domain of the fusion protein acts as a molecular Trojan horse, which crosses the BBB via transport on the endogenous insulin receptor. The avidin domain of the fusion protein creates a high-affinity linker between the HIRMAb and the biotinylated peptide radiopharmaceutical. The 4 kDa Aβ(1−40) amyloid peptide of AD was N-biotinylated and radiolabeled with ^{125}I. The amyloid plaque binding of the [^{125}I]-N-biotinyl-Aβ(1−40) peptide, either without or with conjugation to the HIRMAb-AV fusion protein, was tested with film autoradiography and tissue sections of autopsy AD brain. The study shows the biotinyl-Aβ(1−40) peptide still binds to amyloid plaque in AD brain to the same extent when the peptide radiopharmaceutical is either free or conjugated to the HIRMAb-AV fusion protein. The study supports further evaluation of antibody-targeted peptide radiopharmaceuticals as large molecule neuro-imaging agents that penetrate the BBB.



INTRODUCTION

The dementia of Alzheimer's disease (AD) correlates with the accumulation in the brain of amyloid plaque formed by the Aβ$^{1-42/43}$ amyloid peptide.[1,2] Amyloid forms via a process of nucleation-dependent polymerization,[3] and radiolabeled forms of the amyloid peptide, such as Aβ$^{1-40}$, image amyloid plaque in tissue sections of AD brain with film autoradiography.[4] Aβ peptide radiopharmaceuticals have not been developed as *in vivo* neuro-imaging agents, because the water-soluble Aβ peptide does not cross the blood-brain barrier (BBB).[5,6] Peptide radiopharmaceuticals, such as the Aβ peptide, can be reformulated for transport across the BBB with the combined use of molecular Trojan horse and avidin−biotin technologies. The Aβ$^{1-40}$ peptide was mono-biotinylated and coupled to a fusion protein of avidin (AV), and a genetically engineered chimeric monoclonal antibody (mAb) against the mouse transferrin receptor (TfR), designated the cTfRMAb-AV fusion protein.[6] The cTfRMAb binds the BBB TfR, and this triggers receptor-mediated transport across the BBB of the cTfRMAb and the Aβ$^{1-40}$ peptide radiopharmaceutical, which is joined to the mAb via a biotin-AV linker. Following intravenous (IV) injection in the mouse, the brain uptake of the [^{125}I]-N-biotinyl]Aβ$^{1-40}$ peptide was very low, 0.1% injected dose (ID)/gram brain.[6] Conversely, following conjugation to the cTfRMAb-AV fusion protein, the brain uptake of the peptide radiopharmaceutical was 2.1% ID/g.[6] This level of brain uptake is high, and comparable to the brain uptake of lipid soluble small molecule brain amyloid imaging agents.[7]

The cTfRMAb Trojan horse is specific for the mouse, and cannot be developed as a neuro-imaging agent for humans with AD. A human-specific BBB Trojan horse is the genetically engineered mAb against the human insulin receptor (HIR), and both chimeric and humanized forms of the HIRMAb have been produced.[8] A fusion protein of the engineered HIRMAb and AV was produced in stably transfected Chinese hamster ovary (CHO) cells grown in biotin depleted serum-free medium.[9] The HIRMAb-AV fusion protein is a 200 kDa bifunctional molecule, which both binds the HIR on the human BBB to trigger receptor-mediated penetration of the brain from blood and binds biotinylated peptides, such as the 4 kDa Aβ$^{1-40}$ peptide. Once in brain, the complex of the Aβ$^{1-40}$/HIRMAb has dual binding targets: the Aβ amyloid plaque and the endogenous HIR on brain cells. The objectives of the present study were twofold. First, the binding of the free Aβ$^{1-40}$ peptide vs the Aβ$^{1-40}$/HIRMAb complex to amyloid plaque was evaluated with film autoradiography and frozen sections of AD autopsy brain. Second, the relative binding of the Aβ$^{1-40}$/HIRMAb complex to the amyloid plaque vs binding to the

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endogenous insulin receptor on human brain cells was examined.

EXPERIMENTAL PROCEDURES

Materials. Human Aβ^{1-40} was purchased from Bachem Inc. (Torrance, CA). Biotin-LC-β-Amyloid (1 - 40) (*N*-biotinyl Aβ^{1-40}) was obtained from Anaspec Inc. (Fremont, CA) and Iodine-125 [^{125}I] was purchased from Perkin-Elmer (Waltham, MA). Chloramine T, sodium metabisulfite, and other reagents were purchased from Sigma Aldrich (St. Louis, MO). Snap-frozen human autopsy Alzheimer's disease brain, used for the film autoradiography experiments, was provided by Prof. Harry Vinters (UCLA Department of Pathology, Neuropathology). The HIRMAb-AV fusion protein was produced in stably transfected CHO cells as described previously.[9] The CHO cells were grown for 4 weeks in biotin depleted serum-free medium, and the harvested condition medium was clarified by depth filtration, and the fusion protein was purified by protein A affinity chromatography.[9] The fusion protein was formulated in 0.01 M Tris buffered saline, pH = 5.5, and was stored either sterile filtered at 4 °C or at −70 °C. The murine HIRMAb was produced from the hybridoma with the ascites method and purified by protein G affinity chromatography, as described previously.[10]

Iodination of *N*-biotinyl-Aβ^{1-40}. *N*-biotinyl Aβ^{1-40} (10 ug, 2.1 nmol) was iodinated with ^{125}I (2 mCi; 1.1 nmol) and chloramine T (42 nmol) at room temperature. The reaction was quenched by the addition of sodium metabisulfite (126 nmol), and 100 μL of 0.01 M PBS (0.01 M Na_2HPO_4, 0.15 M NaCl, pH 7.4) containing 1% Tween-20 (PBST) was added to the reaction solution. [^{125}I]-*N*-Biotinyl-Aβ^{1-40} was purified by Sephadex G25 size-exclusion chromatography using PBST as elution buffer. [^{125}I]-*N*-Biotinyl-Aβ^{1-40} had a trichloroacetic acid (TCA) precipitability of >99% and a specific activity of 173 μCi/μg.

Film Autoradiography. Snap-frozen AD brain specimens were embedded in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA), and 20 μm frozen sections were prepared with a Mikron cryostat at −20 °C, thaw-mounted onto glass slides and preserved at −70 °C until use. The slides were warmed to room temperature and briefly air-dried. Slides were then incubated in duplicates with 250 μL of PBSB buffer (0.01 M Na_2HPO_4, 0.15 M NaCl, pH 7.4, 0.1% bovine serum albumin) containing one of several solutions: (a) 1 nM [^{125}I]-*N*-biotinyl-Aβ^{1-40} alone; (b) 1 nM [^{125}I]-*N*-biotinyl-Aβ^{1-40} conjugated with 5 nM HIRMAb-AV; (c) 1 nM [^{125}I]-*N*-biotinyl-Aβ^{1-40} coincubated with 10 μM of unlabeled Aβ^{1-40}; (d) 1 nM [^{125}I]-*N*-biotinyl-Aβ^{1-40} conjugated with 5 nM HIRMAb-AV and coincubated with 10 μM of unlabeled Aβ^{1-40}; (e) 1 nM [^{125}I]-*N*-biotinyl-Aβ^{1-40} conjugated with 5 nM HIRMAb-AV and coincubated with 100 ug/mL murine HIRMAb; and (f) 1 nM [^{125}I]-*N*-biotinyl-Aβ^{1-40} conjugated with 5 nM HIRMAb-AV and coincubated with 100 ug/mL murine HIRMAb and 10 μM of unlabeled Aβ^{1-40}. The slides were incubated at room temperature for 2 h and washed four times with 2 min washes in PBSB at 4 °C followed by two 5 s washes in H_2O at 4 °C. After air-drying, the slides were placed in opposition to the Kodak Biomax MR X-ray film (Carestream Health, Rochester, MN), and exposed for up to 24 h at −70 °C. The films were then developed, fixed, and washed. Autoradiography images were digitized using a UMAX PowerLook III flatbed scanner and Adobe Photoshop, and quantified by estimating the mean gray matter integrated optical density of 5 randomly selected areas, using NIH *Image* v 1.62.

RESULTS

The [^{125}I]-*N*-biotinyl-Aβ^{1-40} peptide radiopharmaceutical was avidly bound by sections of AD brain (Figure 1A), and this

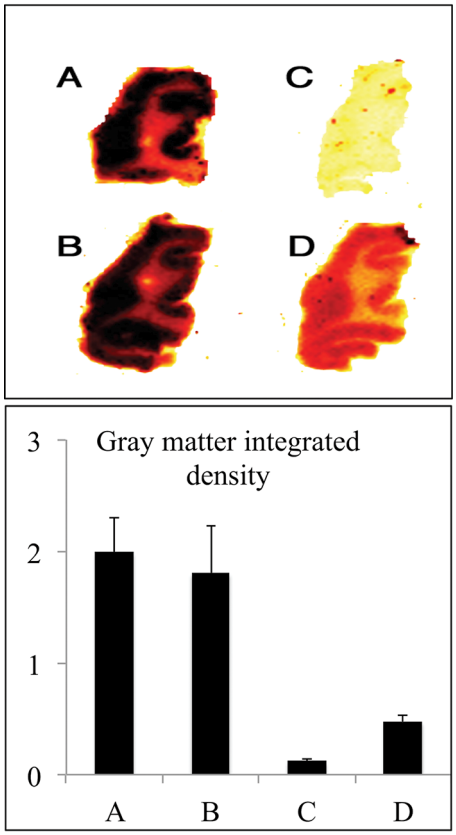

Figure 1. Labeling amyloid plaque in Alzheimer's disease brain with [^{125}I]-*N*-biotinyl Aβ^{1-40} conjugated to HIRMAb-AV fusion protein. (Top panel) Film autoradiograms of human AD brain sections showing the binding of 1 nM [^{125}I]-*N*-biotinyl Aβ^{1-40} coincubated with no additive (A); coincubated with a 5-fold molar excess of the HIRMAb-AV fusion protein (B); coincubated with 10 μM unlabeled Aβ^{1-40} (C); and coincubated with both a 5-fold molar excess of the HIRMAb-AV fusion protein and 10 μM unlabeled Aβ^{1-40} (D). (Bottom panel) Integrated density of radiolabeling over gray matter corresponding to autoradiograms in panels A, B, C, and D. Mean ± SD (n = 5 fields each section).

binding was not altered when the Aβ peptide was first conjugated to the HIRMAb-AV fusion protein (Figure 1B). The binding of the Aβ peptide alone to AD brain was displaced by coincubation of the Aβ with 10 μM unlabeled Aβ^{1-40} (Figure 1C); scanning densitometry shows that the binding of free Aβ peptide is decreased 94% by 10 μM unlabeled Aβ^{1-40} (Figure 1, bottom panel) . There was residual binding of the Aβ/HIRMAb complex to brain in the presence of 10 μM unlabeled Aβ^{1-40} (Figure 1D); scanning densitometry shows the binding of the Aβ/HIRMAb complex is decreased 76% by 10 μM unlabeled Aβ^{1-40} (Figure 1, bottom panel). Therefore, 76% of the Aβ/HIRMAb complex is bound by amyloid plaque in AD brain, and 24% is either bound to the HIR or bound nonspecifically.

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The selective binding of the Aβ/HIRMAb complex to amyloid plaque in AD brain was observed when the Aβ/HIRMAb complex was coincubated with 100 ug/mL HIRMAb (Figure 2A). The HIRMAb alone, without a fused AV domain, preferentially binds the Aβ amyloid plaque of AD brain as compared to the endogenous HIR on brain cells (Figures 1 and 2).

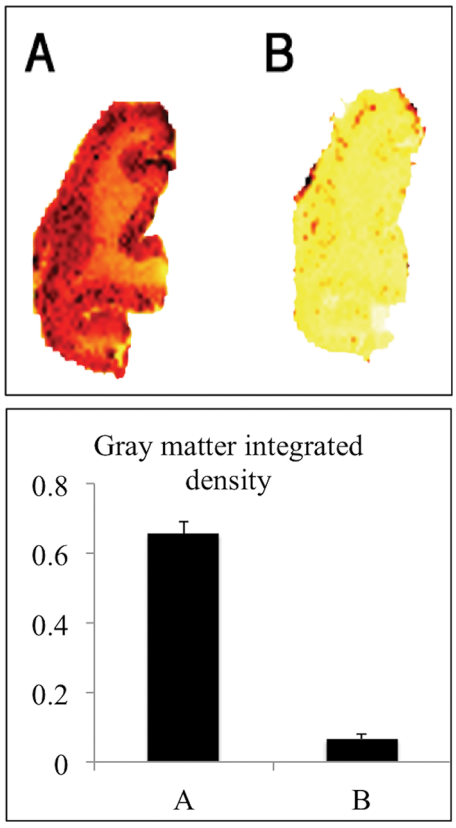

Figure 2. Labeling amyloid plaque in Alzheimer's disease brain with [^{125}I]-*N*-biotinyl Aβ^{1-40} conjugated to HIRMAb-AV fusion protein. (Top panel) Film autoradiograms of human AD brain sections showing the binding of 1 nM [^{125}I]-*N*-biotinyl Aβ^{1-40} coincubated with a 5-fold molar excess of the HIRMAb-AV fusion protein and 100 ug/mL murine HIRMAb (A); coincubated with a 5-fold molar excess of the HIRMAb-AV fusion protein, 100 ug/mL murine HIRMAb, and 10 μM unlabeled Aβ^{1-40} (B). (Bottom panel) Integrated density of radiolabeling over gray matter corresponding to autoradiograms in panels A and B. Mean ± SD (n = 5 fields each section).

suppresses binding to the endogenous HIR on brain cells. Under these conditions, the amyloid plaques in gray matter of AD brain are clearly visible (Figure 2A). The coincubation of the Aβ/HIRMAb complex with both 100 ug/mL HIRMAb and 10 uM unlabeled Aβ^{1-40} causes a 90% reduction in the binding of the Aβ/HIRMAb complex to AD brain (Figure 2B).

DISCUSSION

The results of the present studies are consistent with the following conclusions. First, a new form of Aβ peptide radiopharmaceutical is described, wherein the monobiotinylated Aβ^{1-40} is conjugated to a novel fusion protein of the genetically engineered HIRMAb and avidin. This fusion protein is designated the HIRMAb-AV fusion protein and the complex of biotinylated Aβ^{1-40} to the HIRMAb-AV fusion protein is designated the Aβ/HIRMAb complex. Second, autoradiography of AD brain shows that the Aβ peptide still binds the amyloid plaque of AD brain following conjugation to the HIRMAb-AV fusion protein (Figure 1). Third, the Aβ/HIRMAb complex

Radiolabeled forms of the Aβ peptide are potent agents for detection of amyloid plaque in autopsy sections of AD brain, and the sensitivity of detection of amyloid plaques with the radiolabeled Aβ^{1-40} and autoradiography is far greater than that with an anti-Aβ antibody and immunocytochemistry.[4] The binding of Aβ^{1-40} to amyloid plaques in AD brain is 50% saturated at 30 nM,[4] the binding follows first-order kinetics,[11] and the active binding species is the monomer/dimer, not the oligomer of the Aβ^{1-40} peptide.[12] All of these properties make the Aβ^{1-40} peptide an ideal candidate for drug development as a peptide radiopharmaceutical for imaging the amyloid plaque in AD. However, the IV injection of Aβ peptide radiopharmaceuticals in AD transgenic mice does not image amyloid plaque in brain,[13] because the Aβ peptide does not cross the BBB.[5,6] What is needed is a reformulation of the Aβ peptide radiopharmaceutical as a BBB-penetrating molecule. The Aβ/HIRMAb complex is a new brain-penetrating form of the Aβ peptide radiopharmaceutical. In order for the Aβ/HIRMAb complex to enable effective imaging of the amyloid plaque burden in AD, the imaging agent must have several properties. First, the Aβ/HIRMAb complex must penetrate the brain from blood via receptor-mediated transport on an endogenous BBB receptor. Early studies with the murine HIRMAb showed that Aβ alone did not cross the BBB, but that a complex of Aβ and the murine HIRMAb rapidly penetrated the Rhesus monkey brain *in vivo*.[10] Transport into brain via the BBB insulin receptor allows the imaging agent to penetrate the entire brain volume, including both regions of interest (ROI) bearing amyloid plaque and non-ROI areas with no amyloid plaque. Second, the amyloid binding properties of the Aβ peptide must be retained following reformulation as the Aβ/HIRMAb complex, so that the imaging agent is sequestered within the ROI following penetration into the brain. This is demonstrated by the autoradiography results of the present study (Figures 1 and 2). Third, the Aβ/HIRMAb complex must undergo efflux from brain back to blood, so that the radioactivity is selectively sequestered within the ROI of brain. Prior work in primates shows that the Aβ/HIRMAβ complex does efflux from brain with a half-time of 16 h.[10] The efflux from brain to blood of IgG molecules is mediated by a BBB Fc receptor,[14] and confocal microscopy of brain shows the principal Fc receptor on the BBB is the neonatal Fc receptor, or FcRn.[15] These 3 properties contribute to the successful imaging of amyloid plaque in brain with either large-molecule peptide radiopharmaceuticals, such as Aβ, or lipid-soluble small-molecule amyloid binding agents.[7]

In the absence of BBB drug delivery technology, the brain uptake of Aβ peptide radiopharmaceuticals is very low compared to the brain uptake of small-molecule imaging agents.[7] The brain uptake and biodistribution of the Aβ/HIRMAb complex cannot be assessed in lower animals such as rodents, because the HIRMAb does not cross react with the insulin receptor of lower animals, including New World primates such as squirrel monkeys.[16] However, brain uptake can be assessed in the mouse using a surrogate Trojan horse, the cTfRMAb-AV fusion protein.[6] The brain uptake in the mouse of [^{125}I]-*N*-biotinyl Aβ alone is very low following IV injection, 0.10 ± 0.06% of ID/gram brain.[6] However, following conjugation of [^{125}I]-*N*-biotinyl Aβ to the cTfRMAb-AV fusion protein, the brain uptake in the mouse is high, 2.1% ± 0.2% of ID/gram brain after IV administration,[6] which is comparable to

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the brain uptake of lipid-soluble small-molecule amyloid imaging agents.[7]

The Aβ/HIRMAb complex can be developed as a radiopharmaceutical for either positron emission tomography (PET) or single photon emission computed tomography (SPECT). The 124-iodine nuclide, which has a half-time of 4.2 days, could be incorporated for PET imaging. Alternatively, the 111-indium nuclide, which has a half-time of 2.8 days, could be incorporated for SPECT imaging. Prior work has shown that *N*-biotinyl Aβ^{1-40} can be conjugated with an ^{111}In-chelator moiety, and this modification does not alter peptide binding to amyloid plaque in AD brain.[17]

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Notes

The authors declare the following competing financial interest(s): Dr. Boado is an employee and Dr. Pardridge is a consultant to ArmaGen Technologies.

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