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Lynnae M. Patrias

Andrea C. Klaver

Mary P. Coffey

John M. Finke

University of Washington Tacoma, jfinke@uw.edu

Jyothi L. Digambaranath

See next page for additional authors

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Authors

Lynnae M. Patrias, Andrea C. Klaver, Mary P. Coffey, John M. Finke, Jyothi L. Digambaranath, Loan Dang, Alvaro A. Martinez, and David A. Loeffler

Effects of External Beam Radiation on *In Vitro* Formation of Abeta1-42 Fibrils and Preformed Fibrils

Lynnae M. Patrias,^a Andrea C. Klaver,^a Mary P. Coffey,^b John M. Finke,^c Jyothi L. Digambaranath,^c Loan Dang,^d Alvaro A. Martinez^e and David A. Loeffler^{a,1}

Departments of ^a Neurology Research, ^b Biostatistics and ^c Radiation Oncology, William Beaumont Hospital Research Institute, Royal Oak, Michigan 48073; and ^c Department of Chemistry and ^d Eye Research Institute, Oakland University, Rochester, Michigan 48309

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Plaques containing fibrillar amyloid-beta (Abeta) are a characteristic finding in Alzheimer's disease. Although plaque counts correlate poorly with the extent of cognitive deficits in this disorder, fibrillar Abeta can promote neuronal damage through a variety of mechanisms. External beam radiotherapy has been reported to be an effective treatment for tracheobronchial amyloidosis, in which amyloid is deposited as submucosal plaques and tumor-like masses in the trachea and/or bronchi. Radiotherapy's effectiveness in this disorder is thought to be due to its toxicity to plasma cells, but direct effects of radiotherapy on amyloid may also be involved. On this basis, whole-brain radiotherapy has been suggested as a treatment for Alzheimer's disease. The objective of this study was to determine the effects of external beam radiation on preformed Abeta1-42 fibrils and on the formation of these fibrils. Using the Thioflavin-T assay, no effects of radiation were found on either of these parameters. Our results in this *in vitro* study suggest that whole-brain irradiation is unlikely to directly reduce plaque counts in the Alzheimer's disease brain. This treatment might still lower plaque counts indirectly, but any potential benefits would need to be weighed against its possible neurotoxic effects, which could induce further cognitive deficits. © 2011 by Radiation Research Society

INTRODUCTION

Amyloid-beta (A β), a 4.5-kDa peptide produced by cleavage of amyloid precursor protein (APP), is the main component of senile plaques in the Alzheimer's disease (AD) brain. Plaques and neurofibrillary tangles (NFT) are the hallmark pathological findings in the AD brain. The "amyloid hypothesis," as originally proposed, suggested that increased expression and deposition of

A β in the brain precedes the processes such as inflammation, oxidative stress, NFT formation and neuronal loss that are thought to result in AD-related cognitive impairment (1). However, this hypothesis was difficult to reconcile with many studies indicating that plaque counts are weakly correlated with the extent of dementia in AD patients (2–8); in fact, some individuals with high plaque loads have apparently normal cognition (9–12). A similar "disconnect" between memory and A β deposition was reported in the Tg2576 (Swedish mutation) amyloid precursor protein (APP) transgenic mouse model of AD; in a combined group of old and young Tg2576 mice, there was no obvious correspondence between spatial reference memory and insoluble A β (13). A β has become the main target for AD immunotherapy, although concerns have been expressed that it may not be an appropriate target (14). Recent therapeutic strategies for AD have included vaccination with A β (15), administration of a "humanized" mouse anti-A β monoclonal antibody (16), and inhibition of the amyloidogenic pathway of APP (17). Thus far these efforts have not been successful. The amyloid hypothesis has been modified to reflect current thinking that soluble aggregates of A β , termed A β soluble oligomers, rather than plaque-associated fibrillar A β , may initiate the sequence of pathological events leading to AD-type dementia (18, 19). This modified hypothesis is supported by the recent finding that when synthetic A β 1-42 oligomers were injected into the cerebral ventricles of young C57BL/6 mice, immediate short-term memory impairment occurred, whereas this effect was not seen when A β 1-42 fibrils or freshly dissolved A β 1-42 was injected (20).

Several studies have reported external beam radiotherapy to be an effective treatment for tracheobronchial amyloidosis (TBA), a rare condition in which amyloid is deposited in the trachea and bronchi as submucosal plaques and/or tumor-like masses (21–24). The amyloid in TBA is thought to result from excessive production of immunoglobulin light chains by plasma cells in the tracheobronchial tree (25, 26). The mechanism by which

¹ Address for correspondence: William Beaumont Hospital Research Institute, 3811 West Thirteen Mile Road, Royal Oak, MI 48073; e-mail: DLoeffler@beaumont.edu.

radiotherapy reduces the amyloid deposits in this disorder may therefore involve radiation-induced damage to these plasma cells, which are reported to be radiosensitive (27, 28). Additional mechanisms suggested to account for radiotherapy's effectiveness against TBA include direct degradation of amyloid, an increase in amyloid clearance, and/or damage to the local vasculature (21, 24, 29). On this basis, whole-brain irradiation has been proposed as a treatment for AD (29). Surprisingly, a literature search found no studies of the effects of external beam radiation on A β , although extensive laser irradiation was reported to destroy preformed A β fibrils (30). The objective of this investigation was therefore to determine the effects of radiation on preformed A β 1-42 fibrils and on the development of these fibrils.

MATERIALS AND METHODS

Disaggregation of A β

A β 1-42 (0.5 mg; AnaSpec, San Jose, CA) was disaggregated by resuspending in 0.25 ml trifluoroacetic acid (FA, Sigma-Aldrich, Inc., St. Louis, MO) followed by hexafluoro-2-propanol (Sigma-Aldrich). It was divided into aliquots in eppitubes (20 μ l/tube), dried overnight (16–20 h) at room temperature in the fume hood, and stored at -20° C. Total protein measurement (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA) indicated that each tube contained approximately 10.8 μ g of A β .

Western Blot

Western blotting was performed to confirm that the disaggregation of A β 1-42 performed in our laboratory would produce an apparently pure monomer preparation (e.g., without detectable aggregates) with which to initiate the growth of A β fibrils. Western blots were performed on A β specimens subjected to gel electrophoresis under both reducing/denaturing and native conditions. The procedure for the reducing/denaturing gel electrophoresis was reported previously (31). Native gels were generated by electrophoresing A β through a 4–20% Tris-HCl Ready Gel (Bio-Rad). Twenty microliters of an A β monomer preparation [6 μ g/ml, prepared as described previously (31); total amount added per lane, 0.12 μ g] was mixed with an equal volume of Native Sample Buffer (Bio-Rad), then loaded into the appropriate lane; 14-kDa, 66-kDa and 132-kDa standards from Sigma-Aldrich's Non-Denaturing Molecular Weight Kit (catalog no. MWND500) were run in additional lanes. The transfer of proteins to the PVDF membrane and subsequent staining with mouse monoclonal 6E10 (anti-A β 1-16; Covance Research Laboratories, Berkeley, CA) were the same as for the reducing/denaturing gels.

Production of A β Fibrils: General Procedure

A β 1-42 was resuspended in DMSO (Sigma-Aldrich) to a concentration of 8.1 mg/ml using three eppitubes of previously disaggregated A β . Four microliters of DMSO was added to the first eppitube and water bath-sonicated for 10 min. This was then transferred to the second tube, and after sonication to the third tube, where it was again sonicated. The A β preparation was then diluted to 100 μ M (450 μ g/ml) by adding 68 μ l of 10 mM HCl, then incubated at 37° C and assayed for Thioflavin-T reactivity as described below. A growth curve for A β fibril formation was initially performed in which Thioflavin-T reactivity was measured in the A β preparation and in the negative control tube

(DMSO/HCl) immediately after preparation (day 0) and on days 4 and 7. Based on the results from this experiment, fibrils were grown for 7 days in subsequent experiments.

Irradiation of A β Fibrils

The effects of radiation on preformed A β fibrils (produced as described above) and on the formation of these fibrils were examined in four types of experiments. Initial experiments involved exposure of preformed A β fibrils to 10 Gy, with the Thioflavin-T assay performed immediately thereafter. Subsequent dose-response experiments were performed in a similar manner except that A β fibrils were exposed to either 5 or 20 Gy. The longer-term effects of radiation on preformed A β fibrils were then examined by irradiating preformed fibrils with 10 Gy, then continuing the incubation of the A β preparations at 37° C for an additional 2 or 4 weeks prior to performing the Thioflavin-T assay. The final type of experiment investigated the influence of radiation on the formation of A β fibrils; A β monomer preparations were irradiated at time 0 with 10 Gy, then incubated in fibril-promoting conditions for 1 week, followed by the Thioflavin-T assay. The negative controls for all experiments were sham-irradiated A β samples and vehicle controls (DMSO/HCl) without A β . Each type of experiment was performed on at least three separate occasions. The term "repetition," where used below, will refer to a separate performance of a type of experiment.

Thioflavin-T Assay

Thioflavin-T (Acros Organics, Fairlawn, NJ) was prepared as a 0.25 mM stock solution (pH 6) in 100 mM sodium phosphate and 100 mM sodium chloride. The solution was stored at 4° C, then allowed to reach room temperature on the day of the experiment. It was mixed at a 25:1 ratio with the A β fibril sample. After vortexing, the mixture sat at room temperature for 30 min, then was mixed by trituration and divided into aliquots (100 μ l per well) in multiple wells of a black opaque 96-well culture plate (PerkinElmer, Waltham, MA). The plate was read at 37° C with excitation at 450 nm and absorption at 485 nm on a Molecular Devices SpectraMAX Gemini EM microplate spectrofluorometer (Molecular Devices Corp., Sunnyvale, CA). Softmax Pro Version 5.0 (Molecular Devices) was used to evaluate the data. The Thioflavin-T reactivity of A β fibril preparations in each experiment was measured as relative fluorescence units; in experiments in which the effects of radiation on fibrils were examined, the data were expressed as a percentage of the reactivity of the vehicle control. All samples were tested in a blinded fashion.

Transmission Electron Microscopy (TEM)

A β fibrils were grown for 7 days as described above; then 10 Gy-irradiated and sham-irradiated A β fibril preparations as well as an A β monomer preparation were imaged by TEM using methods described previously (32). Each sample was spread on a Formvar-coated grid (Electron Microscopy Sciences, Fort Washington, PA) and incubated for 2 h at room temperature, then rinsed with double-distilled water. Samples were then fixed with 1% glutaraldehyde in 100 mM phosphate buffer, pH 7.4, for 10 min, rinsed again with water, and stained with 1% uranyl acetate for 10 min and alkaline lead citrate for 5 min. Images were taken with a Morgagni 268 transmission electron microscope (FEI Company, Hillsboro, OR) equipped with a Hamamatsu digital camera. The TEM operator was blinded to the identities of the samples.

Statistics

For statistical analysis of the A β fibril growth curve, comparisons of Thioflavin-T reactivity between A β -containing and negative

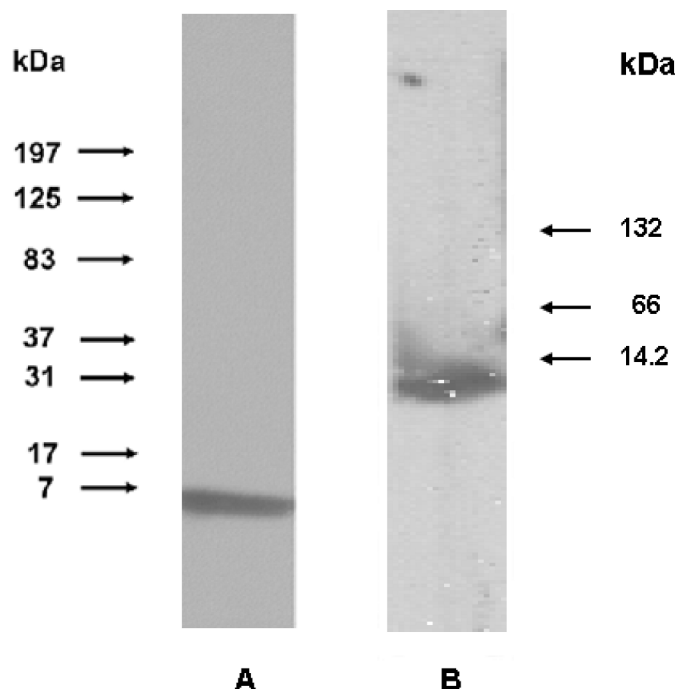


FIG. 1. Western blots of Aβ1-42 after disaggregation with trifluoroacetic acid and hexafluoro-2-propanol prior to its incubation in fibril-promoting conditions. Lane A, reducing/denaturing gel; lane B, native gel. Only one band is seen on each gel. The distances of migration for molecular weight standards are shown for each of the gels; individual nondenaturing molecular weight standards from Sigma-Aldrich were used with the native gel. Total protein loaded: lane A, 0.20 μg; lane B, 0.12 μg.

control (DMSO/HCl) tubes were performed on days 0, 4 and 7 by two-sample *t* tests. For experiments in which the effects of radiation were examined on preformed Aβ fibrils or on the development of these fibrils, the distribution of the results from each experiment set was first analyzed to determine if it met the assumptions of the proposed statistical tests. The assumption of a normal distribution was assessed with normal probability plots and the Anderson-Darling statistic while the assumption of equal variances was assessed by data plots, the Brown-Forsythe test and the Levene test. These analyses were performed for the normalized data, which were obtained by dividing the relative fluorescence units data by the mean of the negative (vehicle-only) controls for a given experiment. Standard linear mixed models using restricted maximum likelihood estimation were used to assess treatment effects when the data were reasonably consistent with its assumptions (i.e., normal distribution and equal variances); when there was evidence of unequal variances across treatments and/or repetitions of an experiment, modifications to the mixed-model ANOVA that adjust for unequal variances were considered. For this study, treatment (vehicle controls, irradiation at a specified dose, and sham irradiation) was considered fixed while each repetition of an experiment and the treatment-experimental repetition interaction were considered random. Least-squares treatment means with their appropriate standard errors summarized the overall response to each treatment in each set of experiments. Tukey-Kramer *P* values and confidence intervals were used for multiple comparisons of treatments for the normalized data from the dose-response experiments. Statistical significance for all analyses was determined using the 0.05 level. The SAS System for Windows version 9.2 and Minitab Release 14 were used for this analysis.

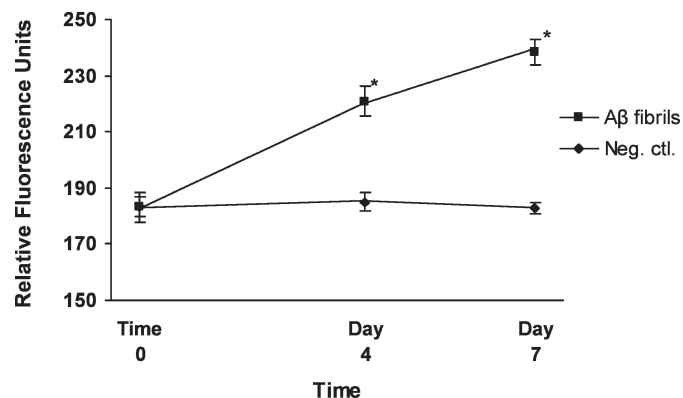


FIG. 2. Growth curve for Aβ1-42 fibrils. Thioflavin-T reactivity is shown as relative fluorescence units for Aβ1-42 incubated in fibril-promoting conditions and for a vehicle (DMSO/HCl) control. Reactivity was evaluated immediately after resuspending Aβ (day 0) and at 4 and 7 days. *n* = 3–4 wells per time; **P* < 0.05 with two-sample *t* test compared to MSO/HCl control.

RESULTS

Western Blots

Western blots of the freshly disaggregated Aβ used for growing fibrils revealed one band, presumably corresponding to Aβ monomer, in both reducing/denaturing and native gels (Fig. 1).

Aβ Fibril Growth Curve

Thioflavin-T reactivity of the Aβ fibril preparation was significantly different from the DMSO/HCl control on both day 4 (*P* = 0.004) and day 7 (*P* < 0.001) (Fig. 2).

Influence of Radiation on Preformed Aβ Fibrils: Acute Studies

The effects of radiation were initially examined on preformed (7-day incubated) Aβ fibrils by irradiating the fibrils with 10 Gy, followed immediately by measurement of Thioflavin-T reactivity. Because preliminary analysis showed unequal variances, the variance of the assay results was allowed to vary for each repetition of the experiment. The means ± SEM for Thioflavin-T reactivity of irradiated and sham-irradiated fibrils, normalized for background fluorescence of the vehicle control, are shown in Fig. 3a. There was no significant difference in Thioflavin-T reactivity between the two groups [*P* = 0.35; 95% CI for irradiated mean – sham-irradiated mean: (–17.27, 7.25)].

A dose-response study was then performed in which preformed Aβ fibrils were exposed to 0 Gy (sham irradiation), 5 Gy or 20 Gy and then Thioflavin-T reactivity was evaluated. The experiment was performed on three separate occasions. There was no evidence of a difference in mean normalized Thioflavin-T reactivity among the three groups (*P* = 0.817). All of the adjusted *P* values for Tukey pairwise comparisons were at least 0.80 [95% Tukey CI for differences: 5 Gy – 20 Gy:

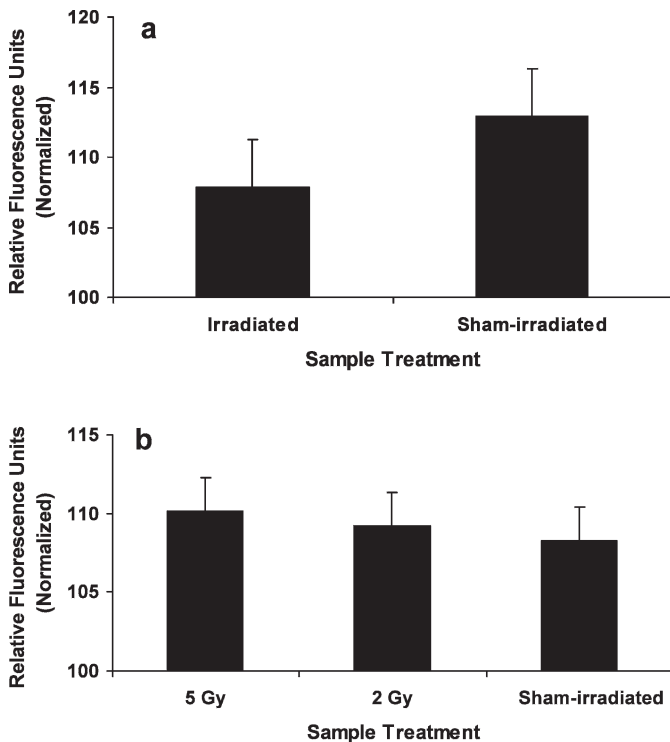


FIG. 3. Influence of radiation on preformed A β 1-42 fibrils: acute studies. The effects of radiation on preformed (7-day) A β fibrils were examined. Panel a shows Thioflavin-T reactivity of irradiated and sham-irradiated fibrils immediately after exposure to 10 Gy, and panel b shows reactivity after exposure to 5 or 20 Gy or sham irradiation. No significant differences in Thioflavin-T reactivity were observed between irradiated and sham-irradiated fibrils. Data shown are least-squares means \pm SEM for normalized values (percentage of vehicle control) and are from four separate repetitions in panel a and three separate repetitions in panel b.

($-8.98, 10.76$); 5 Gy – sham-irradiated: ($-8.06, 11.67$); 20 Gy – sham-irradiated: ($-8.95, 10.78$)]. Data are shown in Fig. 3b.

Influence of Radiation on Preformed A β Fibrils: Longer-Term Studies

Preformed (7-day) A β fibrils were irradiated with 10 Gy or sham-irradiated; then incubation was continued for 2 and 4 weeks before Thioflavin-T reactivity was evaluated on these specimens and on the vehicle-only control. The experiment was performed on three separate occasions. Statistical analyses were performed separately for each period. For week 2 data, in each of the three experiments, the mean of the normalized Thioflavin-T reactivity was larger for the irradiated group than for the sham-irradiated group, but this difference was not statistically significant [$P = 0.114$; 95% CI for 10 Gy – sham-irradiated: ($-3.82, 16.96$)]. For week 4 data, there was no indication of a significant difference in the normalized Thioflavin-T reactivity between irradiated and sham-irradiated samples [$P = 0.68$; 95% CI for 10 Gy – sham-irradiated: ($-7.21, 5.24$)]. Data are shown in Fig. 4.

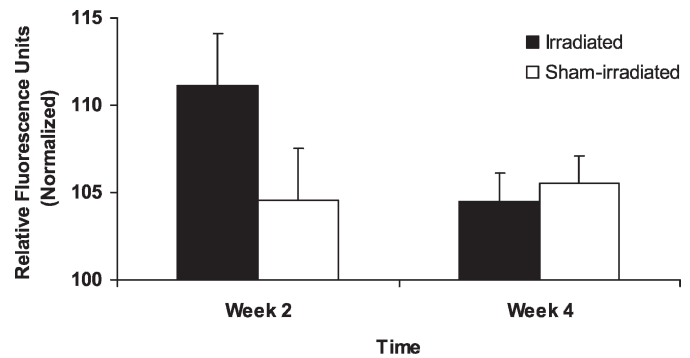


FIG. 4. Influence of radiation on preformed A β 1-42 fibrils: longer-term studies. Preformed (7-day) A β fibrils were irradiated with 10 Gy or sham-irradiated; incubation was continued for 2 and 4 weeks before Thioflavin-T reactivity was evaluated on these specimens and on the vehicle-only control. There were no significant differences in Thioflavin-T reactivity between irradiated and sham-irradiated fibril preparations at either time. Data shown are least-squares means \pm SEM for normalized values (percentage of vehicle control) and are from three separate repetitions of this experiment.

Influence of Radiation on Development of A β Fibrils

Freshly disaggregated A β was irradiated with 10 Gy or sham-irradiated, then incubated in fibril-promoting conditions for 1 week, followed by measurement of Thioflavin-T reactivity. The experiment was performed on five separate occasions. Preliminary examination of the data suggested potential problems with unequal variances across the treatment-experimental repetition combinations; however, using a more complicated variance structure did not improve the analysis, so the standard linear mixed model was used. No significant differences were found in normalized Thioflavin-T reactivity between the irradiated and sham-irradiated A β preparations ($P = 0.62$; 95% CI for irradiated – sham-irradiated: ($-5.21, 7.73$)]. Data are shown in Fig. 5.

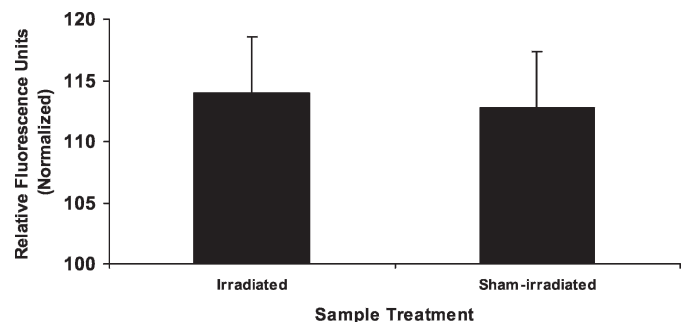


FIG. 5. Influence of radiation on development of A β fibrils. Freshly disaggregated A β was irradiated with 10 Gy or sham-irradiated, then incubated in fibril-promoting conditions for 1 week, and Thioflavin-T reactivity was evaluated. No significant differences were found between irradiated and sham-irradiated A β preparations. Data shown are least-squares means \pm SEM for normalized values (percentage of vehicle control) and are from five separate repetitions of this experiment.

Transmission Electron Microscopy

Extensive fibril formation was observed in both the nonirradiated and irradiated A β fibril preparations. These structures were not seen in the A β monomer preparation. Representative TEMs are shown in Fig. 6a–c.

DISCUSSION

Radiotherapy has been suggested as a potential treatment for AD based on its effectiveness against TBA (29). Although these effects are thought to be primarily due to its toxicity to plasma cells in the trachea or bronchi, direct degradation of A β could also be involved, because radiation's ability to damage proteins is well documented (33–35). In this *in vitro* study, we found no evidence for direct degradation of preformed A β fibrils by external beam radiation or for radiation-induced impairment of A β fibril development.

The Thioflavin-T assay is the most frequently used procedure for monitoring fibril growth (36, 37), and TEM is commonly used to confirm fibril growth. Thioflavin-T reactivity is generally reported in arbitrary fluorescence units, so comparing results between laboratories can be problematic. The dye undergoes a shift in fluorescence after binding to β -pleated sheet structures, but the exact mechanism of Thioflavin-T's binding to amyloid fibrils is unclear (37). The Thioflavin-T reactivity in our A β fibril preparations was often less than 10% greater than the vehicle control, although the presence of fibrils was confirmed by TEM. We therefore performed each experiment on multiple occasions and used a statistical analysis that considered the data obtained on these multiple occasions.

Our negative findings suggest that external beam radiation, when used in our *in vitro* experimental paradigm, does not degrade A β fibrils. However, we cannot rule out the possibility that radiation may have induced alterations in A β below the sensitivity of detection of the Thioflavin-T assay, although we regard this as unlikely. Even if radiation does not directly damage fibrillar A β , it could still reduce plaque counts in the AD brain by indirect mechanisms; for example, radiation-induced inflammation in the brain (38–40) might increase the clearance of fibrillar A β by plaque-associated microglia. Although activated microglia efficiently phagocytose A β fibrils and plaques *in vitro*, they fail to do so in the AD brain (41). However, even if radiotherapy does improve phagocytic removal of plaques, the possible benefits of this approach would need to be weighed against the potentially damaging effects of whole-brain radiotherapy such as brain atrophy (42). Postirradiation complications can include cognitive deficits (42–45), which may take years to develop (46–49) and can be induced by low doses of

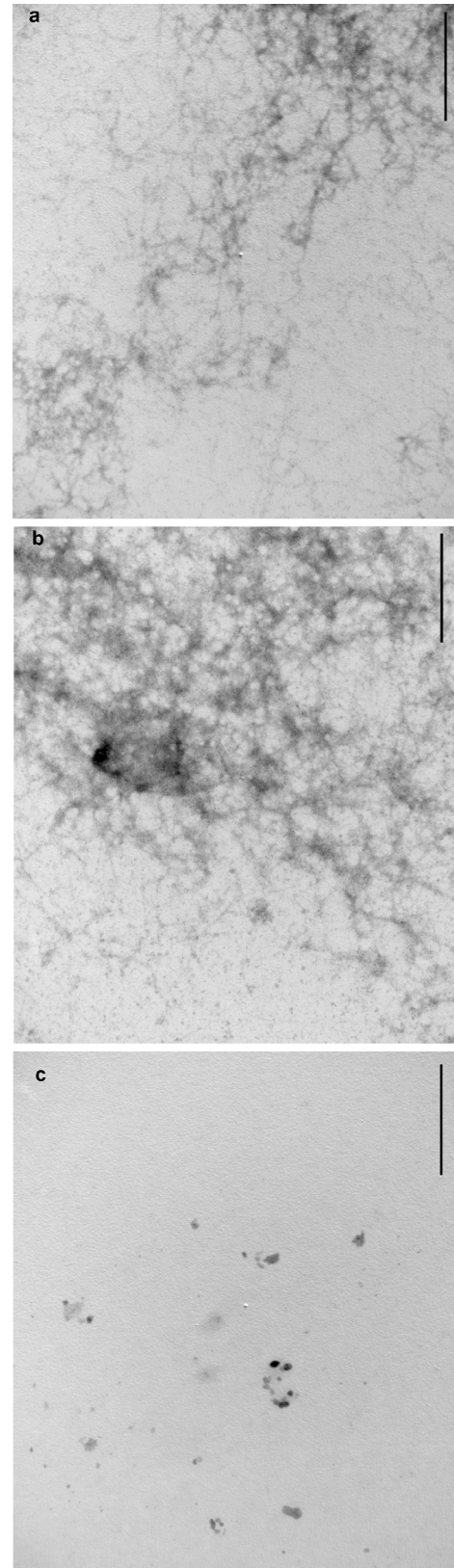


FIG. 6. TEM images. Extensive growth of fibril-like structures was present in both nonirradiated (panel a) and irradiated (panel b) A β fibril preparations. Fibrils were not seen in the A β monomer preparation (panel c). Bar = 500 nm.

radiation even without signs of overt tissue damage (50). The hippocampus, an area of extensive pathology in AD, is particularly sensitive to radiation-induced injury (51); in experimental mice, 10 Gy whole-brain irradiation impaired hippocampal-dependent spatial learning and memory (50, 52). Radiation-induced inflammation in the brain could also be damaging; CNS inflammatory processes are already increased in AD (53), and inflammation has been referred to as a “double-edged sword” in AD and other neurodegenerative disorders because of its potential for both neurotoxic and neuroprotective effects (54).

In conclusion, we found no evidence for direct degradation of A β fibrils or for reduced production of these fibrils by the single doses of external beam radiation that were examined in this *in vitro* study. Although radiotherapy might still reduce plaque counts in the AD brain by indirect means, the potential dangers of this approach would need to be considered.

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