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# β-Amyloid Protein Induces the Formation of Purine Dimers in Cellular DNA

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**Abstract** Experimental evidence implicates oxidative free radical reactions as central in the processes of neurodegenerative diseases. In particular, cellular interactions with the β-amyloid protein have been linked to neuron cell death in Alzheimer's disease. Also, uncharacterized dimeric purine moieties have been detected in oxidized DNAs. It has been suggested that inadequate excision-repair of such products plays a functional role in the neurological degeneration observed in familial Alzheimer's disease, Down's syndrome, and xeroderma pigmentosum. Therefore, in order to obtain a reagent to monitor the presence of such products, the purine dimer 8-8-(2′-deoxyguanosyl)-2′-deoxyguanosine-5′-monophosphate was used as a hapten for elicitation of rabbit anti-purine dimer antiserum. This antiserum specifically recognizes various purified 8-8-bideoxyribonucleosides and 8-8-bideoxyribonucleotides. We found that DNA oxidized by the Fenton reaction is specifically recognized by this antiserum. This reagent can therefore be used to demonstrate formation and excision of DNA purine dimers. Moreover, incubation of cultured rat pheochromocytoma PC-12 cells with the β-amyloid protein resulted in formation of these purine dimers in cellular DNA. These dimers were subsequently removed from cellular DNA. From these results we conclude that the free radicals generated by Aβ cause oxidative DNA alterations including purine dimers. Deficient repair of this type of DNA damage might result in neural cell loss via apoptosis. Our findings suggest mechanisms for the roles of β-amyloid and oxidative free radicals in neurodegenerative diseases and the role of DNA excision-repair in the prevention of lethal neurotoxicity. J. Cell. Biochem. 81:393–400, 2001. 

**Key words:** RAGE; β-amyloid; DNA oxidation; purine dimers; neurodegeneration

DNA repair processes may be central in protecting the cells of the central nervous system from the consequences of DNA oxidation, which, if uncorrected, can result in cell death [Friedberg et al., 1995]. Products of DNA oxidation had not originally been related to the phenotypic and clinical properties of xeroderma pigmentosum, the classic disease with defective nucleotide excision repair, which results in symptoms marked by cutaneous cancers following actinic exposure [Friedberg et al., 1995]. However, a number of properties of xeroderma pigmentosum cells cannot be explained by their known properties, neither by their documented defects in the repair of DNA damages (i.e., those resulting from ultraviolet irradiation or reactions with chemicals that produce large, bulky adducts) nor by their roles in transcription. One particular association of xeroderma pigmentosum combining neurologi-

Abbreviations used: Aβ, β-amyloid protein; APP, amyloid precursor protein; RAGE, receptor for advanced glycation end products; PS1, presenilin 1; PS2, presenilin 2.

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cal defects with dermatological manifestations (the DeSanctis-Cacchione syndrome) involves progressive neurological deterioration with massive neuron loss [Kraemer et al., 1987]. Various neurological abnormalities, frequently accompany xeroderma pigmentosum, with low intelligence, ocular abnormalities and abnormal motor activity being the most prominent [Friedberg et al., 1995; Kraemer et al., 1987]. There is much clinical and laboratory evidence that the neurodegeneration frequently observed in this disease is etiologically related to the DNA repair deficiency in this disease, with resultant accumulation of uncorrected altered DNA moieties [Parshad et al., 1996; Robbins, 1989].

Oxygen free radicals induce formation of DNA lesions not repaired by xeroderma pigmentosum cells [Satoh et al., 1993]. This study used oxidized substrate superhelical DNA from which the major monomeric base damages, including thymine glycol and 8-oxoguanine, had been removed. The reaction conditions were those that allowed complete excision repair of DNA damages [Wood et al., 1988]. These experiments demonstrated reduced excision-repair of such superhelical oxidized DNAs by cell extracts from xeroderma pigmentosum groups A, B, C, D, and G [Satoh et al., 1993]. Therefore, oxidation of DNA generates base damages corrected by the nucleotide excision repair pathway, but which are defectively repaired in xeroderma pigmentosum cells. A previous study indicated formation of uncharacterized dimeric purine moieties in oxidized DNAs [Carmichael et al., 1992]. While the classic form of DNA damage studied in these cells is the far-UV-induced cyclobutane pyrimidine dimer, the neurodegeneration observed in xeroderma pigmentosum patients obviously cannot be due to this photoproduct.

Other studies indicate a major role for oxidative processes in the neurodegeneration of Alzheimer's disease [Good et al., 1996; Pappolla et al., 1998]. The major lesions involve neurofibrillary tangles and senile plaques, both in insoluble aggregations [Selkoe, 1996]. One of the early pathological changes in Alzheimer's disease involves extracellular accumulation of the 39–43 amino acid β-amyloid protein (Aβ), eventuating in its cerebral deposition [Scheuner et al., 1996]. Many other changes have been described, and, while it is as yet undetermined which changes are the primary causes of this disease, a major role for Aβ appears likely. Mutations in three genes (APP, PS1, PS2) result in increased deposition of Aβ [Citron et al., 1997]. In particular, mutations in the APP gene encoding the protein that is proteolytically digested to Aβ are associated with familial Alzheimer's disease, with increased Aβ secreted from cells that result in deposition of the highly amyloidogenic residues of 42 or 43 amino acids often detected in senile plaques [Selkoe et al., 1996]. The cellular toxicity of Aβ protein is mediated by hydrogen peroxide [Behl et al., 1994]. In addition, linkage between Aβ deposition and the generation of oxidative free radicals has been demonstrated in cultured rat pheochromocytoma PC-12 cells [Yan et al., 1996]. Aβ reacts with the receptor for advanced glycation end products (RAGE). This results in generation of intracellular reactive oxygen intermediates [Yan et al., 1996]. These intermediates have not been characterized, but such compounds are capable of oxidative modification of DNA, which, if unrepaired, could result in neural cell death. A recent in vivo study demonstrated that blockade of the RAGE results in reduction of amyloid-induced cellular dysfunction [Yan et al., 2000]. Taken together, these studies suggest possible mechanistic linkages between Aβ deposition, generation of oxidative free radicals, and the cell death of Alzheimer's disease.

Other work has indicated a major role for DNA repair in protection of the cell from such damage. Unlike the DNA damages induced by ultraviolet irradiation at 254 nm, cellular DNA modifications by fluorescent irradiation are predominantly the indirect result of free radical generation [Demple and Harrison, 1994]. One study demonstrated an increased incidence of DNA chromatid breaks induced by fluorescent irradiation and subsequent treatment by either β-cytosine arabinoside or caffeine in cultured cells from patients with a number of neurodegenerative diseases, including familial (but not sporadic) Alzheimer's disease, Down's syndrome, and complementation group A of xeroderma pigmentosum [Parshad et al., 1996]. Therefore, a type of oxidative DNA damage is induced that is inadequately repaired in these cells. Such products are substrates for the nucleotide excision repair system [Satoh et al., 1993]. In view of these findings, it is logical that elucidation of the modes of induction and extent of excision of

dimeric DNA oxidative lesions can significantly enhance our understanding of the molecular bases of human neurological degenerative disease.

## METHODS

### Elicitation of Antiserum to DNA Purine Dimers

The dipurinyl dehydrodimer 8-8-(2′-deoxyguanosyl)-2′-deoxyguanosine-5′-monophosphate was synthesized and purified as described [Krishnamachary et al., 1995]. It was used as the hapten for elicitation of the rabbit anti-purine dimer antiserum. This purine dimer was coupled to bovine serum albumin in the presence of 1-ethyl-3-diisopropylaminocarbimide-HCl, injected into rabbits with Freund's adjuvant to elicit anti-purine dimer antiserum, which was obtained and lyophilized [Harlow and Lane, 1988].

### Detection of Purine Dimers in Oxidized DNA

In the Fenton reactions, denatured calf thymus DNA were oxidized at a concentration of 400 μg/ml by 50 mM $H_2O_2$ in the presence of either 100 μM $CuSO_4$ or $FeSO_4$ at 37°C for 15 or 30 min; this yields about 42 diverse oxidized moieties per $10^8$ bases [Carmichael et al., 1992]. Denatured calf thymus DNA was oxidized, with modification of 15% of the thymines, by reaction with 1.5 mM $KMnO_4$ at 4°C for 5 min as described [Frenkel et al., 1981]. Preparation of the dot blot followed the method of Harlow and Lane [1988]. 100 μg of DNA was bound by slow filtration to nitrocellulose paper, which was dried for 2 h in a vacuum oven and blocked overnight in 5% BSA. The primary antibody solution was 10 ml of a 1:350 dilution of a 1 mg/ml stock solution of lyophilized rabbit anti-purine dimer antiserum. The Sigma ExtrAvidin kit, which contains biotinylated goat anti-rabbit IgG, avidin-coupled alkaline phosphatase, and Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetra-zolium tablets were then used to process the immunoblot according to the instructions of the manufacturer (Sigma, St. Louis, MO).

### Detection of DNA Purine Dimers in PC-12 Cells Incubated with Aβ

Rat pheochromocytoma PC-12 cells (ATCC) were grown and incubated in 1 mM rat Aβ (1–42; Calbiochem.) for 4 h according to Yan et al. [1996]. DNAs were purified using the PERFECTgDNA Genomic DNA Preparation System (5 Prime–3 Prime, Inc.). Two μg of cellular DNA were placed on each spot on nitrocellulose paper and bound by slow filtration. The DNA was denatured on a Whatman No. 1 paper soaked in 1.5 M NaCl, 0.5 N NaOH for 10 min at room temperature and renatured on a Whatman No. 1 paper soaked in 1 M NaCl, 0.5 M Tris–HCl, pH 7.0 for 5 min at room temperature. The blot was dried for 2 h in a vacuum oven and blocked overnight in 5% BSA. The primary antibody solution was 10 ml of a 1:350 dilution of a 1 mg/ml stock solution of lyophilized rabbit anti-purine dimer antiserum. The Sigma ExtrAvidin kit, which contains biotinylated goat anti-rabbit IgG, avidin-coupled alkaline phosphatase, and Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets were then used to process the immunoblot according to the instructions of the manufacturer (Sigma, St. Louis, MO).

## RESULTS

The 8-8-dipurinyl dehydrodimer, 8-8-(2′-deoxyguanosyl)-2′-deoxyguanosine-5′-mono-phosphate, was synthesized, purified, and used as a hapten for elicitation of rabbit anti-purine dimer antiserum [Krishnamachary et al., 1995]. The structure of this product is shown in Figure 1. A dot-blot assay was used to detect antiserum recognizing damaged DNA immobilized on nitrocellulose membranes. The rabbit anti-purine dimer antiserum recognized all purified 8-8-bideoxyribonucleosides and 8-8-bideoxyribonucleotides in this assay (data not shown). These include 8-8-dipurinyl dehydrodimers involving two guanines, two adenines, or a guanine and an adenine. The Fenton reaction causes the induction of uncharacterized

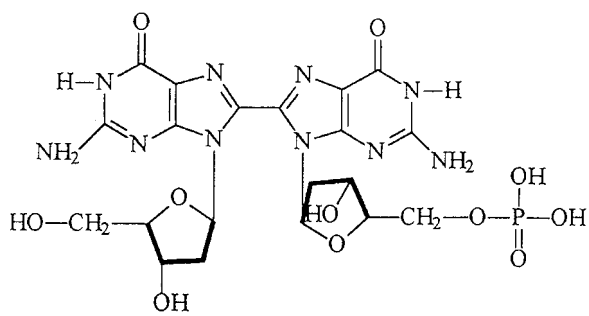

**Fig. 1.** Structure of the hapten: the DNA purine dimer 8-8-(2′-deoxyguanosyl)-2′-deoxyguanosine-5′-monophosphate.

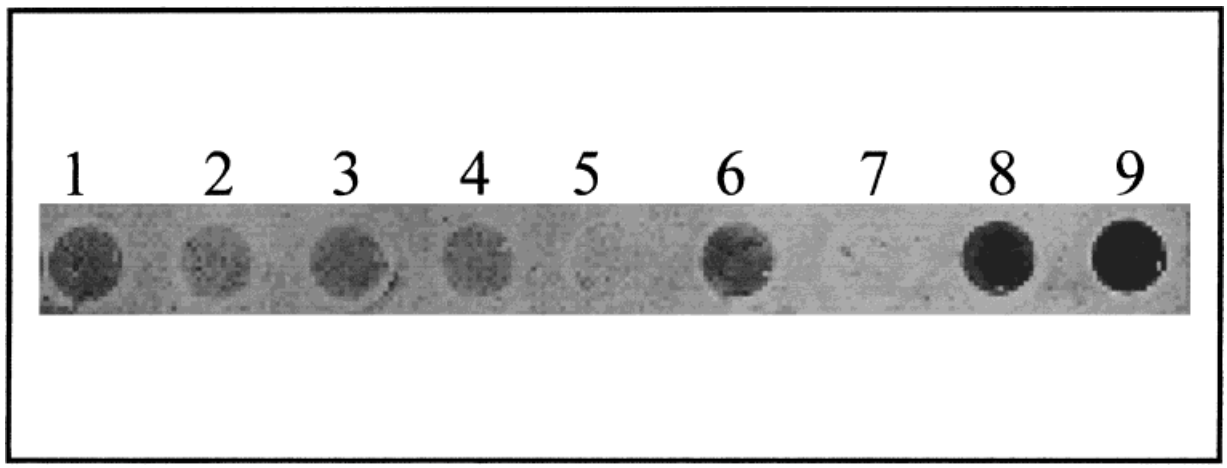


**Fig. 2.** Immunological detection of purine dimers in DNA oxidized by the Fenton reaction. Denatured calf thymus DNA was oxidized at a concentration of by 50 mM $H_2O_2$ in the presence of either $CuSO_4$ or $FeSO_4$ at 37°C for 15 or 30 min. This yields about 42 oxidized moieties per $10^8$ bases [Carmichael et al., 1992]. Oxidation of denatured calf thymus DNA by was $KMnO_4$ at 4°C for 5 min. This results in oxidation of 15% of the DNA thymines [Frenkel et al., 1981]. Preparation of the dot blot followed the method of Harlow and Lane [1988]. **Dots**: 1, DNA oxidized by $CuSO_4$ for 15 min; 2, DNA oxidized by $CuSO_4$ for 30 min; 3, DNA oxidized by $FeSO_4$ for 15 min; 4, DNA oxidized by $FeSO_4$ for 30 min; 5, DNA oxidized by $KMnO_4$; 6, the purine dimer 8-8-(2′-deoxyguanosyl)-2′-deoxyguanosine-5′-monophosphate; 7, unoxidized control DNA; 8 and 9, blotted rabbit serum.

dimeric purine products in DNA [Carmichael et al., 1992]. The results of this assay using such oxidized DNAs are shown in Figure 2. The anti-purine dimer antiserum did not bind to unreacted DNA. However, DNAs oxidized by the Fenton reaction (modifying both purines and pyrimidines) are detected by the antiserum. This is independent of the metal ion used, whether $Cu^{2+}$ or $Fe^{2+}$. This demonstrates the affinity of the antiserum for an oxidized DNA moiety. By contrast, DNAs oxidized by other protocols were not detected by the anti-purine dimer antiserum. This includes oxidation by potassium permanganate, which modifies pyrimidines at the 5,6-double bond [Frenkel et al., 1981]. DNA irradiated with visible light in the presence of methylene blue was also unreactive with the antiserum (data not shown). The latter treatment induces specific oxidative purine modifications, with 8-oxoguanine as the major product and minor quantities of formamidopyrimidines i.e., purines with opened imidazole rings [Tuite and Kelley, 1994]. This demonstrates the antiserum is unreactive to DNA 8-oxoguanines, formamidopyrimidines, or oxidized pyrimidines, but reacts with purine dimers. It therefore can be used as a for the study of the induction and excision of purine dimers in cellular DNA.

In order to determine if Aβ can induce damage in cellular DNA of the type that might be implicated in cell death in neurodegenerative diseases, the induction of DNA purine dimers was assayed in cultured PC-12 cells. Since these rat pheochromocytoma cells express the RAGE, incubation with Aβ can result in intracellular free radical generation [Yan et al., 1996]. As shown in Table I, treatment with 1

**TABLE I. Survival of PC-12 Cells Incubated with β-Amyloid Protein[a]**

| | Untreated cells | | Treated cells | |
|---|---|---|---|---|
| Time of incubation | Cell number | Viability | Cell number | Viability |
| 0 h | $2 \times 10^6$ | 98% | $2 \times 10^6$ | 98% |
| 24 h | $3.8 \times 10^6$ | 95% | $4.1 \times 10^6$ | 96% |
| 48 h | $8.1 \times 10^6$ | 97% | $8.3 \times 10^6$ | 97% |

[a]PC-12 cells were incubated in 1 mM β-amyloid protein as described. The cell number was determined by direct cell counting using a hemocytometer. Cell viability was determined by staining with trypan blue.

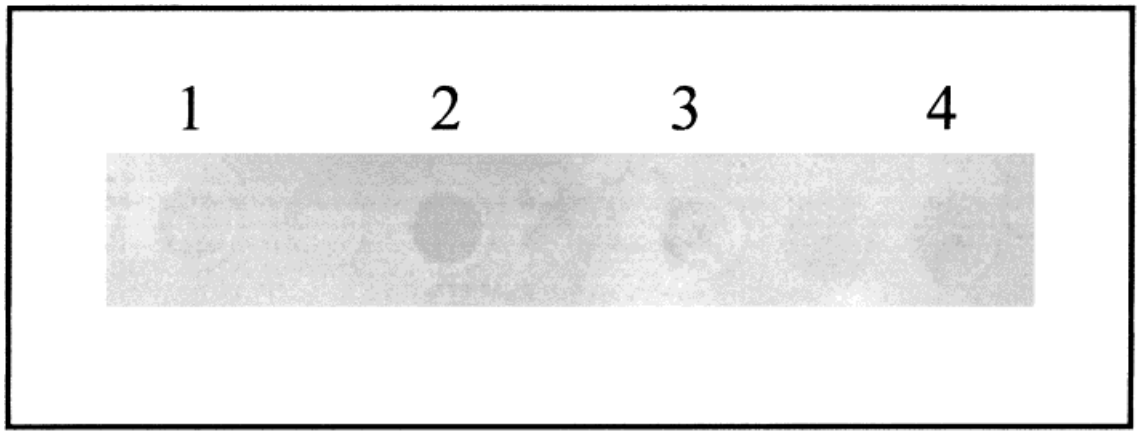


**Fig. 3.** Formation of purine dimers in, and their excision from, DNA of PC-12 cells incubated with β-amyloid protein. PC-12 cells were grown and incubated in 1 mM rat Aβ (1–42; Calbiochem) for 4 h according to Yan et al. [1996]. The DNAs were purified and dot blot assays performed as described. **Dots**: 1, DNA of PC-12 cells; 2, DNA of PC-12 cells incubated in 1 mM Aβ for 4 h; 3, DNA of PC-12 cells washed and incubated for an additional 16 h; 4, DNA of PC-12 cells incubated in 1 mM Aβ for 4 h, washed, and incubated for an additional 16 h.

mM Aβ of up to 48 h did not alter cell viability. PC-12 cells were then grown and incubated with 1 mM rat Aβ (1–42; Calbiochem) for 4 h. One aliquot was lysed for instant analysis; another was washed thoroughly with PBS and incubated in fresh medium for 16 h to determine if DNA damage was removed. The results are shown in Figure 3. The DNA of cells not exposed to Aβ was unreactive to the antiserum. Incubation of the cells in Aβ resulted in the induction of purine dimers in DNA, as detected reaction with anti-purine dimer antiserum. The DNA of Aβ-treated cells, incubated in fresh medium for 16 h after treatment to allow for repair, was unreactive to the antibody. Therefore, incubation of the cells with Aβ results in induction of DNA purine dimers, which are subsequently removed from the DNA.

## DISCUSSION

This study is the first demonstration of a DNA modification, specifically purine dimers, or any DNA damage, in cultured cells resulting from exposure to Aβ. While such exposure has been reported to generate intracellular free radicals, possible resultant damages to DNA had not been studied. Moreover, this type of alteration is removed from DNA via cellular repair mechanisms. A structure such as the 8-8-dipurinyl dehydrodimer studied here would induce a major distortion into DNA. This type of distorting moiety would be a substrate for removal through the nucleotide excision repair pathway [Friedberg et al., 1995]. It is this pathway that is defective in xeroderma pigmentosum, a disease with observed neurodegeneration. It remains to be determined if a defect in the excision of DNA purine dimers can be established in cultured cells from this disease. However, it has recently been demonstrated that another type of minor oxidation product, i.e., the 5′-8-purine cyclodeoxynucleoside, are removed by the nucleotide excision repair system [Kuraoka et al., 2000]. Those results and our experiments both imply that the classical model of oxidative DNA damages as exclusively removed by the base excision repair system is not valid. The two classes of oxidative DNA modifications that are substrates for nucleotide excision repair, i.e., 5′-8-purine cyclodeoxynucleosides and 8-8-dipurinyl dehydrodimers, are not among the major products by chemical yields, but may have potential for major biological significance.

Taken together, these results suggest the emergence of a paradigm for the etiogenesis of a number of human neurodegenerative diseases. Diverse damages to DNA result from oxygen radicals generated from endogenous metabolic processes or from cellular interactions with Aβ. The yields of such modified DNA moieties may vary with the generation of oxygen radicals in specific cell types. However, most moieties are corrected by the base or nucleotide excision-repair systems. However, in a number of diseases, defects are present in the nucleotide excision repair pathway. These would include familial Alzheimer's disease, Down's syndrome and xeroderma pigmento-

sum [Parshad et al., 1996]. Such DNA repair defects would result in persistence of minor types of unrepaired oxidative DNA damages, possible 5′-8-purine cyclodeoxynucleosides and 8-8-dipurinyl dehydrodimers, which eventually results in cell death. Since neurons are permanent cells that cannot reproduce, the cell loss cannot be replenished and pathological sequelae inevitably ensue. These sequelae result in the classic clinical and pathological presentations of these various neurodegenerative diseases.

The possible mechanisms of neural cell loss involve a possible major role of unrepaired oxidative DNA base damages, possibly the purine dimers studied here, in the etiogenesis of neurodegeneration. Such modifications may be related to a prominent finding in Alzheimer's disease, i.e., intracerebral amyloid plaques [Selkoe et al., 1996]. This is consistent with a model derived from in vivo studies of transgenic mice [Pappolla et al., 1998]. It involves initial abnormalities resulting in a nidus for precipitation, extracellular accumulation, and eventual cerebral deposition of Aβ [Scheuner et al., 1996]. A wide variety of abnormalities may result in this initial injury. Such factors may include repeated brain trauma, such as in dementia pugilistica [Mortimer et al., 1991; Tokuda et al., 1991]. These eventually result in enhanced amyloidogenesis, such as present in those mutations in the presenilin or APP genes [Pappolla et al., 1998]. Such mutations are significant risk factors for Alzheimer's disease, resulting in increased cerebral deposition of Aβ [Citron et al., 1997; Lamb, 1997]. This may be abetted by defective removal of amyloid peptides, especially in patients with specific apolipoprotein E polymorphisms or in the presence of pathologic chaperones [Pappolla et al., 1998]. Once deposited, the accumulated Aβ triggers generation of intracellular free radicals through interaction with the RAGE [Yan et al., 1996].

Other factors are consistent with a role for the RAGE in the progression of a chronic neurodegeneration. Unlike other receptors, the RAGE is not down-regulated in the presence of ligand, but rather stimulated [Li and Schmidt, 1997; Yan et al., 1999]. This upregulation of the RAGE by ligands results in a positive feedback loop, with continuous generation of intracellular oxidative free radicals. Therefore, it is possible that even transient interaction between Aβ and the RAGE, present on neural cell surfaces, can eventuate in a cascade of continuous free radicals resulting in oxidative DNA modifications. This would result in various oxidative stress responses, possibly resulting in other observed features of Alzheimer's disease [Pappolla et al., 1998]. These would include neurofibrillogenesis, modification of protein tau, and an increase in protein ubiquitinilation [Pappolla et al., 1998]. One study indicates increased nuclear DNA oxidation in brains from Alzheimer's disease patients, consistent with the possibility of increased oxidative stress [Gabbita et al., 1998]. Unrepaired DNA damage from oxidative free radicals could then result in neuronal cell death. Most of those products are monomeric and therefore repaired by the base excision repair system [Demple and Harrison, 1994; Parshad et al., 1996]. However, interaction of cells with Aβ results in the induction of DNA purine dimers. Should these persist in DNA, either through defects in the nucleotide excision repair system or by the overwhelming of cellular repair capacity, these moieties could result in neural cell death via apoptosis [Satoh et al., 1993]. There is mounting evidence for neural cell loss through apoptosis, rather than necrosis, in Alzheimer's disease [Cotman and Anderson, 1995; Kim et al., 1997; Lamb, 1997; Morrison and Hof, 1997; Ratan et al., 1995; Roperch et al., 1998; Yang et al., 1998]. Therefore, these results suggest a possible mechanistic linkage between Aβ deposition, generation of oxidative free radicals, DNA oxidative modifications including purine dimers, and the neural cell death observed in Alzheimer's disease. The persistent DNA purine dimer might therefore be a molecular link between the presence of Aβ deposits, generation of intracellular free radicals, neural cell loss via apoptosis, and the clinical presentation and course of Alzheimer's disease and other types of neurodegeneration.

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