The involvement of dityrosine crosslinks in lipofuscin accumulation in Alzheimer’s disease

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Abstract. Lipofuscin is hydrophobic and insoluble yellow-brown pigment that accumulates in the nervous system of individuals and considered to be a biomarker of aging. However, it has been reported that amyloid-containing Alzheimer neurons contain a large amount of lipofuscin and lysosomal protease enzymes suggesting that the accumulation of Aβ may contribute to lipofuscin formation under oxidative stress conditions. Probing the contribution of oxidative stress using dityrosine cross-links as a marker will help to raise our understanding of the mechanism underlying the increased lipofuscin accumulation in Alzheimer (AD). In order to establish whether oxidised Aβ42 is found in lipofuscin pigments in AD brains, immunogold labelling for dityrosine and Aβ42 in lipofuscin of AD and control age matched brains was carried out. Single immunogold labelling of dityrosine was observed in the lipofuscin granules of control age-matched human brain. Interestingly, TEM immunogold labelling of dityrosine in lipofuscin of AD brain reveals two different labelling areas, low- and high-density dityrosine labelling. The quantification of immunogold particles shows significantly more dityrosine labelling in AD brain compared to age-matched controls. TEM immunogold co-labelling of dityrosine and Aβ in AD brain reveals some colocalisation within lipofuscin, although some areas showed low levels of Aβ labelling. These results may indicate that dityrosine cross-links could be generated from oxidation of various proteins that contain tyrosine residues. Our data show that dityrosine cross-links are increased in lipofuscin in AD brain, highlighting the important role played by dityrosine cross-links in the accumulation of lipofuscin in higher levels in AD compared to control brains.

Keywords: Alzheimer’s disease; Lipofuscin; dityrosine; amyloid beta; oxidative stress.

Introduction

Alzheimer’s disease (AD) is the most common cause of dementia and is characterised by two pathological hallmarks, extracellular plaques and intracellular neurofibrillary tangles. In AD, amyloid beta (Aβ) misfolds and assembles into insoluble amyloid fibrils which are deposited in extracellular plaques. However, what makes Aβ misfolded is not well known. High oxidative stress has been observed and metal ions, especially copper, have been shown to be involved in the pathogenesis of AD [1]. Oxidative stress is believed to be one of the pathological mechanisms of amyloid plaque formation. On the other hand, it has been suggested that following the death of neurons, lipofuscin...
releases into the extracellular space and this may substantially contribute to the formation of senile plaques. In 1912 the term "lipofuscin" was used for the first time by Hueck [2], which originates from Greek *lipo* (for fat) and Latin *fuscus* (for dark). It is a complex matrix of oxidised proteins and lipid peroxidation products, along with lesser amounts of carbohydrates and metals [3-5]. Iron is the major component of the metal content in lipofuscin [6], also copper and zinc have been found in lipofuscin [7, 8]. Proteomic and ultrastructural analysis of human lipofuscin has identified over 160 proteins and revealed a heterogeneous core structure composed of lipofuscin granules which are surrounded by substantial extra-granular material [9]. Lipofuscin is hydrophobic and insoluble yellow-brown pigment that accumulates in the nervous system of animals and it is more abundant in aged individuals than in young ones, therefore it is called "aging pigment" [3, 10, 11]. Lipofuscin is considered to be a biomarker of aging and the age-dependent accumulation of lipofuscin in the nervous system of individuals has made reliable estimation of absolute age possible. Under the light microscope, lipofuscin is observed as a homogeneously dark mass with irregular shape and surrounded by a 100 nm thick lysosomal membrane [12].

There is a large body of evidence that confirms the involvement of oxidative stress in lipofuscin pigment accumulation over age progression. It seems that the autophagocytosis of mitochondria is the major contributor to lipofuscin formation (Fig1) [13]. Although ROS, such as hydrogen peroxide, superoxide anion, and hydroxyl radical, can be formed from different in vivo sources (e.g. auto-oxidation, photochemical, and enzymatic reactions), mitochondria represent the main in vivo source of ROS formation [14, 15]. At the same time, mitochondria is the main target of ROS attack [16], resulting in the formation of peroxided, undegradable macromolecules. Recently, Murakami and Shimizu have clarified the role of cytoplasmic superoxide radical as a possible contributing factor to intracellular Aβ oligomerisation in AD [17]. It also has been suggested that intraneuronal Aβ oligomers cause neuronal death by activating endoplasmic reticulum stress, endosomal/lysosomal leakage and mitochondria dysfunction [18]. Hydrogen peroxide, an example of ROS, can easily diffuse into lysosomes and react with iron ions that released from the degradation of different metalloproteins during their intralysosomal degradation. The interaction of hydrogen peroxide with iron ions results in the formation of the highly reactive hydroxyl radicals. The latter would attack intralysosomal macromolecules, such as Aβ, causing cross-linking of these materials and leading to lipofuscin formation (Fig1). The oxidative modification, especially cross-linking of autophagocytosed material, is a probable cause of the non-degradability of these materials.

![Figure 1: Schematic diagram of lipofuscin formation in the lysosome. In mitochondria, hydrogen peroxide is generated from superoxide anions, a by-product of mitochondria respiration. In the presence of ferrous ions, hydrogen peroxide can be converted into hydroxyl radicals that attack surrounding macromolecules and leads to oxidative modification. Red dots represent the oxidatively damaged undegradable macromolecules. Adapted from [19].](image-url)
The excessive accumulation of these non-digested materials could result in endosomal/lysosomal leakage and as a consequence, hydrolases enzymes will release resulting in cell death. In support of this hypothesis, it has been shown that the inhibition of lysosomal proteases can dramatically enhance the effect of oxidative stress on lipofuscin formation, leading to delay in the degradation of autophagocytosed substances and allowing more time for its oxidation [10]. On the other hand, lipofuscin accumulation has been shown to be retarded by the iron chelator desferrioxamine, and many antioxidants including vitamin E, selenium, and glutathione [3]. Vitamin E deficiency is associated with increased lipofuscin accumulation [20]. Under oxidative stress conditions, Aβ can undergo many oxidative modifications, one of them is dityrosine cross linking. High levels of dityrosine crosslinks has been found in plaques in AD brain, and it seems to increase the stability of amyloid fibrils [21]. Also, high levels of dityrosine were found in CSF from AD patients, suggesting the possibility of using dityrosine as biomarker for oxidative stress in AD [21]. Dityrosine crosslinks were found in Lewy bodies in Parkinson’s disease and are suggested to play an important role in alpha-synuclein assembly [22]. In this study, immunogold labelling transmission electron microscopy was used as approach to examine the prevalence of dityrosine crosslinking in the lipofuscin in AD patients.

Results and discussion

Immunogold labelling for dityrosine was carried out on embedded thin sections of brain tissue from three AD patients and two age-matched controls to establish whether crosslinked Aβ42 is found in lipofuscin pigments in AD brains. 80 TEM micrographs (40 control and 40 AD) were analysed and linkage density per µm² was determined using in house software. Quantification analysis of gold particles showed the presence of dityrosine in the lipofuscin granules of control age-matched human brain (Figure -2 a & b), indicating the possibility of dityrosine contribution in lipofuscin formation. Interestingly, TEM immunogold labelling of dityrosine in lipofuscin of AD brain reveals two different labelling areas, low-density dityrosine labelling and high density dityrosine labelling (Figure -2, a, inserts in bottom panel), indicating different levels of oxidative stress may have occurred and leading to the variety of dityrosine content that accumulates in lipofuscin. Additionally, it could be due to a variety of proteins that contribute to lipofuscin accumulation. In total, the quantification of immunogold particles show a significant difference (P value <0.001) between dityrosine labelling in AD brain and age-matched control (Figure -2, b). High levels of dityrosine linkage (linkage density = 21.71 linkage/µm²) was observed in AD brain compared to control (linkage density = 8.44 linkage/µm²). Data shows a significant increase of dityrosine level in lipofuscin in brain tissue of AD patients compared to healthy aged matched controls.

![Figure -2: Immungold TEM of lipofuscin in brain tissue of AD patients compared to age-matched controls.](image)

(a) Micrographs reveal single labelling for dityrosine at low levels in lipofuscin in control brains and high levels in AD brain. (b) Quantification of dityrosine cross linkage shows significantly (P value < 0.001) more dityrosine labelling in AD brain compared to age-matched controls.
These results are in good agreement with a previous study using immunohistochemical detection [23], which showed the presence of dityrosine in the lipofuscin granules of the pyramidal neurons in aged human brains from patients with no history of neurological diseases. However, here we find that comparison of AD and age-matched controls revealed a significant increase in density of dityrosine labelling in lipofuscin in AD brains, compared to control (Figure -2, b). These results agree with the capacity of oxidative stress to induce protein modifications resulting in the formation of intralysosomal lipofuscin.

High levels of dityrosine crosslinks have been reported in amyloid plaques in AD brain tissue [21]. In order to determine whether dityrosine resulted from the oxidative modification of Aβ in lipofuscin and to examine the co-localisation of dityrosine and Aβ within lipofuscin pigments, immunogold co-labelling for dityrosine and Aβ in lipofuscin of AD and control age-matched brains was carried out. TEM immunogold co-labelling of dityrosine (15 nm particles) and Aβ (5 nm particles) in AD brain reveals some colocalisation within lipofuscin (Figure 3, a) although some areas showed low levels of Aβ labelling. Quantification analysis was conducted for one micrograph using in house software (Figure 3, b), providing an image whereby particles of dityrosine crosslinks were red and particles of Aβ were blue. The image reveals two different areas of labelling, one shows delocalisation of dityrosine and Aβ (Figure 3, b Top panel), and the second area shows colocalisation of dityrosine and Aβ (Figure 3, b Bottom panel), indicating that dityrosine crosslinks can produce from various proteins that accumulate to form lipofuscin.

Figure 3: Immunogold labelling TEM of lipofuscin in AD brains. a) Double labelling with anti-Aβ (5 nm) and anti-dityrosine (15 nm) reveals colocalisation within lipofuscin from AD brains. b) Particle size analysis for immunogold labelling of lipofuscin in AD brain.
The protease resistant feature of dityrosine that has been reported in many studies [24-26] supports the view that dityrosine can contribute to lipofuscin formation. In our hands, dityrosine crosslinks were found in lipofuscin in control individuals and this is consistent with other results that used immunohistochemical techniques to assess dityrosine level in lipofuscin of healthy aged-matched individuals [23].

Interestingly, the data presented in this study shows significant increased dityrosine level in lipofuscin in brain tissue of AD patients compared to healthy aged-matched controls and that may reflect the important role that is played by oxidative stress in lipofuscin and plaque formation and subsequently AD pathogenesis. High levels of lipofuscin are observed in AD brain tissue, thus it is important to understand the mechanism by which lipofuscin is produced and accumulates in brain tissue. Both lipofuscin and plaques could form as a result of oxidative stress effects. Our findings point to a relationship between dityrosine and amyloid accumulation in the AD brain and support strongly the hypothesis of dityrosine contribution to AD pathogenesis.

It has been suggested that intraneuronal lipofuscin is intrinsically harmful to neuronal cells [12]. On the other hand, Giaccone et al. discussed the role of lipofuscin in the pathogenesis of AD, and proposed that lipofuscin could be harmful to neuronal cells after its release into the extracellular space [27]. However, they reported that lipofuscin contains both Aβ and its precursor and suggested that upon its release into the extracellular space it may serve as a source of Aβ oligomers for a prolonged period. Results presented here support this hypothesis, showing that lipofuscin in AD brain contains both Aβ and dityrosine.

**Conclusion**

Our findings show that dityrosine cross-links were increased in lipofuscin in AD brain, indicating the important role played by dityrosine cross-links in the accumulation of lipofuscin in higher levels in AD compared to control people.

**Materials and methods**

**Brain Samples**

Five AD brains and two aged-matched controls were used in this study as shown in the table below. AD and aged-matched control brain from middle frontal gyrus tissues were obtained from London Neurodegenerative Diseases Brain Bank. Tissue was removed according to Local Ethics Committee guidelines, and informed consent for brain donation was obtained from the next of kin and stored at –80 °C until required.

| Case   | Age | Sex | Pathological diagnosis                                      |
|--------|-----|-----|-------------------------------------------------------------|
| Control 1 | 89  | F   | Control with Hypoxic-type changes and amyloid angiopathy    |
| Control 2 | 80  | F   | Control-minimal ageing changes                              |
| AD 1   | 93  | F   | Alzheimer’s disease HP-tau stage 6 with moderate amyloid angiopathy |
| AD 2   | 86  | F   | Alzheimer’s disease HP-tau stage 6 with mild amyloid angiopathy |
| AD 3   | 77  | F   | Alzheimer’s disease -modified Braak BNE stage 5             |

**Brain tissue blocks preparation**

A section of around 1 mm³ from the frozen brain tissue was cut using a scalpel blade and immediately moved into an Eppendorf tube containing Fixative Solution (4% formaldehyde, 0.1% glutaraldehyde,
PBS 1X) and left overnight at 4°C. The fixative solution was removed and the tissue was washed with buffer (PBS 1X) five times over a four hours period. After washing, the sample underwent dehydration steps via increasing ethanol gradation solutions (30%, 50%, 75%, 90%, 3x 100%; 20 minutes each). After the last 100% Ethanol step, the sample was then prepared for embedding in resin (UNICRYL, BBI Solutions) by incubation in a 2:1 100% ethanol: resin solution for 2 hours, and then in a 1:2 ratio for 30 minutes before moving into complete resin overnight at 4°C. The following day fresh resin was exchanged and the sample was moved into a BEEM capsule (Agar Scientific). Resin polymerization was performed under UV light for 3-4 days at 4°C. The finished block was then removed from the BEEM capsule and prepared for ultra-thin sectioning.

**Ultra-thin Sectioning**

Leica EM UC7 ultramicrotome was used to section the polymerized tissue blocks. The tissue was located in the block and an area of trapezoidal shape was cut using a razor blade (Astra superior platinum double edge razor blade). In order to remove the superficial layer of resin present on the tissue a glass knife was initially used, then ultra-thin sections of 60 nm were cut using a diamond knife (Ultra Diamond Knife - Wet 45° 2.5mm). Sections were collected on hexagonal 300-mesh Nickel grids (3.05 mm, Agar Scientific) for immunogold labelling.

**Immunogold labelling TEM**

The immunogold labelling was performed used well established protocol [28]. A modified phosphate-buffered saline, pH 8.2 (1% BSA, 500 µL Tween-20, 10 mM Na EDTA, and 0.2 g/l NaN₃ (henceforward termed PBS+) was used for all dilutions and rinsing. Briefly, thin brain sections on the supporting grids were incubated with normal goat serum (1:10 dilution) for 30 min at room temperature to block non-specific secondary antibody binding. In turn, grids were labelled with (10 µg/ml IgG) anti-dityrosine mouse monoclonal antibody (Japan Institute for the Control of Aging JICA, Shizuoka, Japan) or double-labelled using a mixture of (10 µg/ml IgG) anti-Aβ42 rabbit polyclonal antibody AB5078P (Chemicon, Temecula, CA, USA) and (10 µg/ml IgG) anti-dityrosine mouse monoclonal antibody and incubated overnight at 4°C. After 3x2 min PBS+ rinses, sections were then immunolabelled with GaM10 or a mixture of GaR5 and GaM15 secondary probes (both 1:10 dilution), respectively, for 1 h at room temperature. After 3x10 min PBS+ and 4x5 min distilled water rinses, the grids were stained in 0.22 µm-filtered 0.5% (w/v) aqueous uranyl acetate for 1 h. The grids were examined on a Hitachi 7100 TEM (Hitachi, Germany) fitted with a Gatan Ultrascan 1000 CCD camera (Gatan, Abingdon, UK), and operating with a voltage of 100 kV.

**Analysis of immunogold labelled sections**

Counting and size analyses of immunogold particle were performed using in house software written by Dr. Kyle Morris in Matlab. Briefly, raw digital electron micrograph images files were uploaded and circular particles detected using inbuilt Matlab circular Hough transforms functions. The detection range diameter was calculated from user input and conversion to pixel size values read from the image header. 15 nm gold particles were distinguished from 5 nm particles based on a user-selected threshold of 7 nm.

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