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Copper Ions Enhance Signal Intensity of Sandwich ELISA for Amorphous Aggregates of Amyloid-β_{42}

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Abstract

Amyloid-β_{42} ($A\beta_{42}$) accumulates within senileplaque, a pathological hall mark of Alzheimer's disease (AD). Our previous reports showed that the monoclonal antibodies 37-11 and 77-3 react with conformational epitopes on the surface of the soluble aggregates of $A\beta_{42}$ and that sandwich ELISA using these two monoclonal antibodies yields high reactivity to detect soluble aggregates of $A\beta_{42}$. Here, the reactivity of the sandwich ELISA was shown to increase in the presence of 50 μM Cu^{2+}. However, the addition of Cu^{2+} had only a small effect on the reactivity of a direct ELISA using antibody 37-11 or 77-3, suggesting that Cu^{2+} has a small effect on the number of epitopes on the surface of the aggregates. Atomic force microscopy images showed that larger aggregates were formed in the presence of Cu^{2+}, as shown in the other reports. Cu^{2+} may gather the aggregates with distinct epitopes recognized by antibodies 37-11 and 77-3, leading to increased signal intensity of the sandwich ELISA.

Keywords

Amyloid-β_{42}, Soluble Aggregates, Antibody, ELISA

1. Introduction

One of major hallmarks of Alzheimer's disease (AD) is the formation of senile plaques, mainly consisting of amyloid beta protein (Aβ), in the brains of patients [1] [2]. Under physiological conditions, Aβ are produced by the partial digestions of amyloid precursor proteins; most Aβs consist of 40 amino acids ($A\beta_{40}$), though approximately 10% are 42 amino acids in length ($A\beta_{42}$) [3]. $A\beta_{42}$ has higher hydrophobicity, and thus a higher tendency to aggregate compared to $A\beta_{40}$; therefore, $A\beta_{42}$ appears to be the more toxic variant [4]. $A\beta_{42}$ forms two distinct aggregates known as fibrillar and amorphous forms.

The fibrillar form has an antiparallel β-sheet structure, and is the main component of senile plaques [5]. The fibril formation is formed from the conversion of monomers to oligomers and subsequently to protofibrils and fibrils [6]. The amorphous aggregates are less structured and lack the ability to form fibrils. They are found in diffuse plaques [7] and senile plaques in the brains of AD patients [8]. Various sizes of amorphous aggregates have been reported ranging from trimers to sub-micrometer orders [9]-[11]. Large oval aggregates (LOA), which are amorphous aggregates greater than 200 nm in their minor axis, are generated by the addition of peptide $A\beta_{16\text{-}20}$ [12]. $A\beta_{16\text{-}20}$ includes the region essential for the fibril formation in Aβ but prevents fibril formation to produce LOAs. As previously described [13], soluble aggregates are prepared by dissolving $A\beta_{42}$ in 1,1,1,3,3,3-hexafluoro-2-propanol in the absence of $A\beta_{16\text{-}20}$. The soluble aggregates include various sizes of amorphous aggregates ranging from 20 to 400 nm. Metal ions such as Cu^{2+} bind to Aβ affecting the formation of aggregates. Many reports show that Cu^{2+} binding changes the surface charge of Aβ and enhances amorphous aggregate formation by preventing fibril formation [14]-[18]. $A\beta_{42}$ in cerebrospinal fluid (CSF) is a clinical biomarker for AD [19] [20]. In AD patients, the concentration of $A\beta_{42}$ decreases, as it is incorporated into senile plaques, whereas the concentration of $A\beta_{40}$ is unchanged. The average concentration of $A\beta_{42}$ in spinal fluid is less than 200 Pm [19]. Therefore, highly sensitive detection of $A\beta_{42}$ is desirable for AD diagnosis. Our previous reports identified various monoclonal antibodies with reactivity against Aβ aggregates, but with little reactivity against the fibril and monomer forms [8] [12] [13] [21]. Among them, antibody 37-11 was determined to react with LOA [12], and antibody 77-3 could react with both LOA and soluble aggregates [13]. Sandwich ELISA using these two antibodies resulted in the highly sensitive detection of soluble aggregates. In this report, the reactivity of the sandwich ELISA was shown to increase through the addition of 50 μM Cu^{2+}; however, the addition had only a small effect on the reactivity of each antibody. Cu^{2+} could gather aggregates to form large aggregates with two distinct epitopes that were specifically recognized by antibodies 77-3 and 37-11.

2. Materials and Methods

2.1. Preparation of the Soluble Aggregates

Soluble amorphous aggregates were prepared as previously described [21]. Briefly, 0.11 mM $A\beta_{42}$ (Peptide Institute Inc., Osaka, Japan) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol, and incubated at 4˚C for 16 h followed by 37˚C for 3 h, after which the solution was lyophilized. The steps including dissolution and lyophilization were repeated twice, and lyophilized $A\beta_{42}$ was dissolved in deionized water. The concentration of $A\beta_{42}$ in the solution was determined by its absorbance at 280 nm using the molar extinction coefficient of 1450 $M^{-1}cm^{-1}$ of $A\beta_{42}$, which has one tyrosine residue and no tryptophan residues.

2.2. Enzyme-Linked Immunosorbent Assay (ELISA)

Direct ELISA was performed as previously described [12]. The soluble aggregates (100

μl) were used as solid-phase antigens, and were stained on 96-well plates (F96 MAXISORP NUNC-IMMUNO PLATE; Thermo Fisher Scientific Inc., Rochester, NY, USA). The monoclonal antibody 37-11 or 77-3, previously conjugated to HRP using a Peroxidase Labeling Kit-SH (Dojindo Molecular Technologies Inc., Kumamoto, Japan), was used as a primary antibody. The substrate SIGMAFAST OPD (Sigma-Aldrich, St. Louis, MO) was used for color development according to the manufacturer's instructions. For sandwich ELISA, the antibody 77-3 was used as the primary antibody, and HRP-conjugated antibody 37-11 was used as the secondary antibody as described previously [13].

2.3. Atomic Force Microscopy

The sizes and shapes of the aggregates were observed by atomic force microscopy (AFM, JSPM-5200, JEOL Ltd, Tokyo, Japan) as previously described [12]. The aggregates (10 μl) were dropped on fresh mica and dried by desiccation, and measured using the altering current mode at room temperature, with a frequency of approximately 190 kHz, typical of resonances with a 4.5 N/m spring constant.

3. Results and Discussion

3.1. Cu^{2+} Increases Signal Intensity of Soluble Aggregates during Sandwich ELISA

Metal ions are known to bind to Aβ and induce the formation of the amorphous aggregates. Metal ions were hypothesized to change the conformation of the epitopes, recognized by antibodies 37-11 and 77-3, and used for the highly sensitive detection of soluble aggregates. Various metal ions (50 μM $MnCl_2$, $FeCl_2$, $NiCl_2$, $CuCl_2$, or $ZnCl_2$) were added to the soluble aggregates and signal intensities were observed after sandwich ELISA using the two antibodies. The addition of $CuSO_4$ increased the signal intensity approximately 9.1-fold; however, the effect of other metal ions had little effect on signal intensities (data not shown). This result is consistent with the previous reports suggesting that binding of Cu^{2+} to Aβ is stronger than that of other metal ions. Thus, the increased signal intensity was considered to be caused by Cu^{2+} binding to Aβ The effect of different concentrations of $CuSO_4$ on the signal intensity of soluble aggregates in the sandwich ELISA is shown in **Figure 1**. The signal increased in the presence of 10 μM $CuSO_4$, and reached a maximum in the presence of $CuSO_4$ ranging in concentration from 25 to 75 μM. These concentrations of Cu^{2+} were much higher than those of Aβ (130 nM in 100 μl), used for the ELISA, and may have induced the formation of amorphous aggregates. Some studies have shown that Cu^{2+} at sub-equimolar metal ion/$Aβ_{42}$ ratios induces the formation of fibrils, whereas supra-molar ratios induce the formation of non-fibrillar forms [15]. In addition, the Cu^{2+} concentrations in this report were similar to those found in the serum (7 - 41 μM) [22]. **Figure 2** shows the effects of $CuSO_4$ and $CuCl_2$ on the signal intensities of the sandwich ELISA. The effect of $CuCl_2$ was greater than that of $CuSO_4$ suggesting that the counter anion also affects signal intensity.

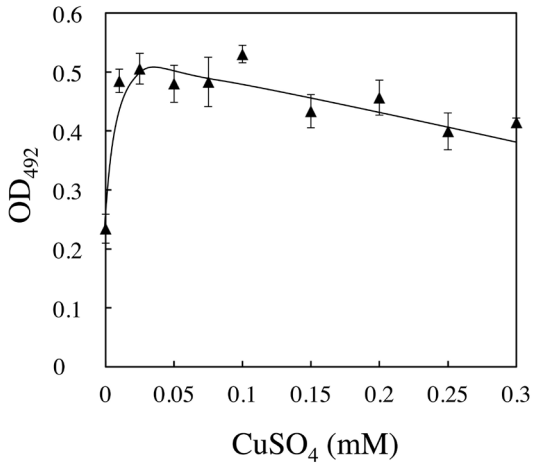


Figure 1. Effect of $CuSO_4$ concentrations on the signal intensity of the sandwich ELISA. Sandwich ELISA was performed using the antibody 77-3 as a primary antibody and HRP-conjugated antibody 37-11 as a secondary antibody. The reactivity of the soluble aggregates (13 pmol), in the presence and absence of various concentrations of Cu^{2+}, was determined.

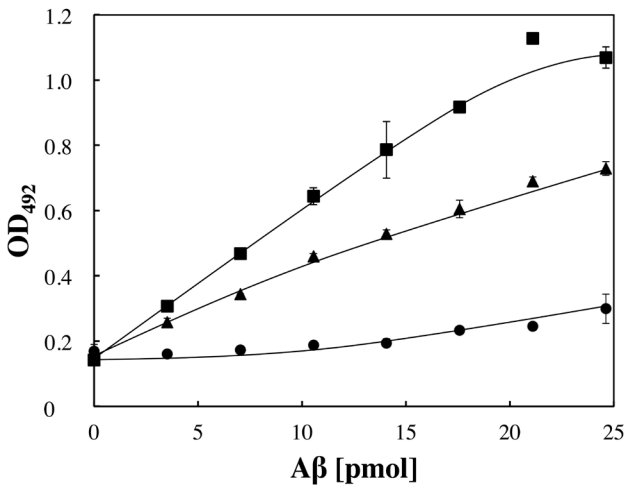
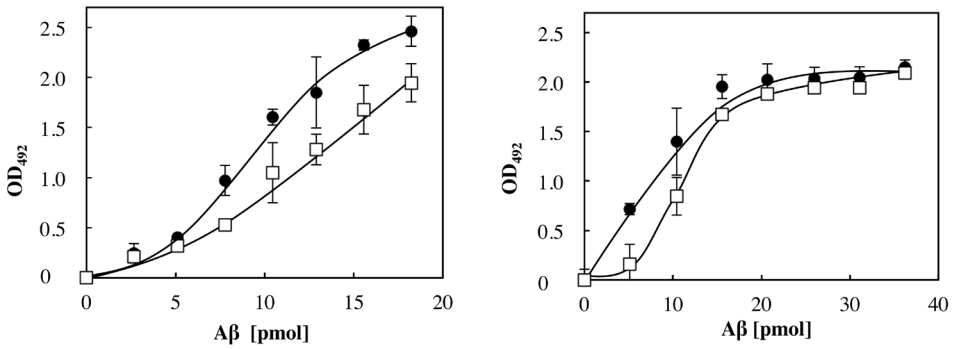


Figure 2. Effect of $CuSO_4$ and $CuCl_2$ on the signal intensity of the sandwich ELISA. Sandwich ELISA using the antibodies 37-11 and 77-3 was performed in the absence (circles) and presence of 50 μM $CuSO_4$ (triangles) or $CuCl_2$ (squares).

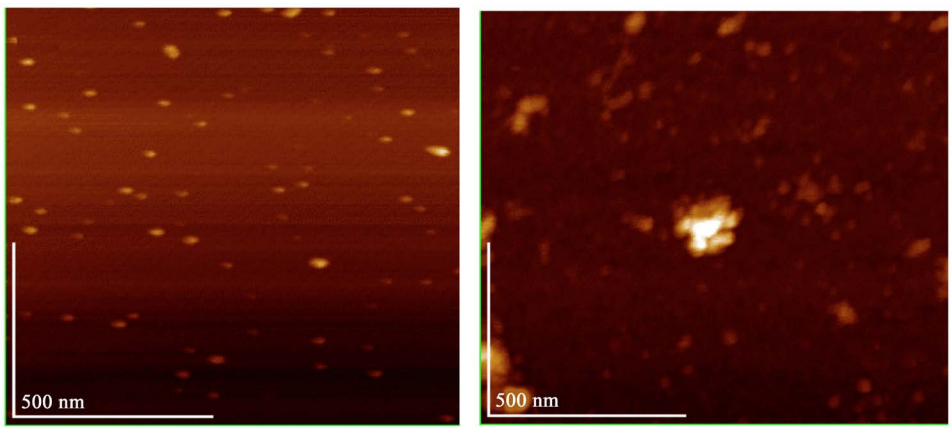
3.2. Cu^{2+} Has Little Effect on the Signal Intensity of the Soluble Aggregates from Direct ELISA

It is possible that the epitopes, recognized by antibodies 37-11 and 77-3, are formed on the surface of soluble aggregates in the presence of Cu^{2+}. To evaluate the formation of new epitopes, direct ELISA was performed. Figure 3 shows the effect of 50 μM $CuCl_2$ on signal intensities. Unlike the results of the sandwich ELISA (Figure 2), Cu^{2+} only minimally enhanced the signal intensity. These results suggest that Cu^{2+} increases the intensity by gathering distinct aggregates with two distinct epitopes recognized by antibodies 37-11 and 77-3, rather than generating these epitopes. The AFM images (Figure 4), showing the formation of larger aggregates through the addition of Cu^{2+}, were



(a) (b)

Figure 3. Effect of $CuCl_2$ on the signal intensity of the direct ELISA. Direct ELISA using HRP-conjugated antibody 37-11 (a) or 77-3 (b) was performed in the absence (open squares) and the presence of 50 μM $CuCl_2$ (closed circles).



(a) (b)

Figure 4. AFM images of soluble aggregates. Soluble aggregates in the absence (a) and presence (b) of 50 μM $CuCl_2$ are shown.

consistent with this theory. Based on this hypothesis, the epitopes recognized by antibodies 37-11 and 77-3 might be separately located on the surface of distinct aggregates, and few aggregates have both epitopes on their surface.

This report shows that the addition of Cu^{2+} increases the reactivity of the sandwich ELISA for soluble aggregates. This finding could contribute to the development of a precise and highly sensitive ELISA for Aβ aggregates by enhancing the reactivity of the antibodies.

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