It has been suggested that amyloid β-peptide (Aβ) might mediate the adhesion of erythrocytes to the endothelium which could disrupt the properties of endothelial cells. We provide evidence here that Aβ actually induced the binding of erythrocytes to endothelial cells and decreased endothelial viability, perhaps by the generation of oxidative and inflammatory stress. These changes are likely to contribute to the pathogenesis of Alzheimer’s disease.

Key words: amyloid β-peptide; red blood cell; endothelial cell; Alzheimer’s disease

The presence of amyloid β-peptide (Aβ) in human blood has recently been established, and it has been hypothesized that Aβ (e.g., Aβ40, Aβ42) readily contacts red blood cells (RBC) and oxidatively impairs RBC functions. We have recently conducted in vitro and in vivo studies which provided evidence that Aβ actually induced oxidative injury to RBC by binding to them, causing RBC phospholipid peroxidation, and diminishing RBC endogenous carotenoids, especially xanthophylls.

Attention has therefore been paid to this type of damage which is likely to injure the vasculature, potentially reducing oxygen delivery to the brain and facilitating Alzheimer’s disease (AD).

With regard to another pathogenic aspect of Aβ in circulating blood, Ravi et al. have proposed that Aβ induced adhesion of RBC to endothelial cells, thereby slowing blood flow and reducing oxygen delivery, especially perhaps in the brain microvasculature with vascular amyloidosis. If Aβ-mediated RBC binding to the endothelium actually occurs, such adhesion may also disrupt the endothelial properties (e.g., viability and functionality). However, this possibility has not previously been evaluated. In this study, RBC were therefore treated or not with Aβ, before being incubated with human umbilical vein endothelial cells (HUVEC). The effects on the adhesion ratio of RBC to HUVEC (the RBC/HUVEC ratio), and the HUVEC viability and function were investigated.

Blood from a healthy male donor (23 years old, body mass index of 19.8 kg/m², blood hemoglobin of 15.5 g/dL, hematocrit of 45.9%, plasma total cholesterol of 158 mg/dL, plasma triacylglycerol of 56 mg/dL, and fasting glucose of 72 mg/dL) was collected in a tube containing EDTA-2Na as an anticoagulant. The donor had no history of medical illness, and gave written informed consent to the experimental protocol which was approved by the ethics committee of Tohoku University. The blood was subjected to centrifugation at 1,000 × g for 10 min at 4 °C. After the plasma and buffy coat had been removed, RBC were washed with phosphate-buffered saline (PBS, pH 7.4) to prepare packed cells (PKC). To these PKC (200 μL), 200 μL of either Aβ (Peptide Institute, Osaka, Japan; 1–10 μM Aβ40 or Aβ42 in PBS) or PBS alone was added. The cells were incubated for 12 h at 37 °C. Aβ-treated and untreated PKC were washed with PBS, counted, and subjected to the subsequent studies with HUVEC.

HUVEC were purchased from Kurabo (Osaka, Japan) and cultured in a HuMedia-EG2 growth medium (Kurabo), which consisted of a base medium (HuMedia-EB2) supplemented with 2% fetal bovine serum (FBS; Dainippon Pharmaceutical, Osaka, Japan), 10 ng/mL of human epidermal growth factor, 50 μg/mL of hydrocortisone, 50 ng/mL of gentamicin, 50 ng/mL of amphotericin B, 5 ng/mL of human basic fibroblast growth factor, and 10 μg/mL of heparin, at 37 °C in a humidified atmosphere containing 5% CO₂. Confluent HUVEC (passages 4–8) were used for the experiments. HUVEC (1 × 10⁵ cells) were seeded on 100-mm dishes and pre-incubated in the HuMedia-EG2 medium for 5 h. After washing HUVEC with PBS, the culture medium was replaced with a test medium (1% FBS/HuMedia-EB2 containing either Aβ-treated or untreated PKC (2 × 10⁶ cells)). After incubating up to 18 h, the cells were washed with PBS and treated with trypsin to liberate RBC and HUVEC. These RBC and HUVEC samples were evaluated with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) to calculate the RBC/HUVEC ratio. A total of 10,000 cells (the sum of RBC and HUVEC) were measured for each sample in the flow cytometric assay.

The effects of Aβ-treated or untreated PKC on HUVEC viability were then evaluated. HUVEC were

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**Note**

**Amyloid β Induces Adhesion of Erythrocytes to Endothelial Cells and Affects Endothelial Viability and Functionality**

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transferred to 96-well plates (1 × 10⁴ cells/well) and pre-incubated in the HuMedia-EG2 medium for five h. After HUVEC had been washed with PBS, the culture medium was replaced with the test medium (1% FBS/HuMedia-EB2 containing either Aβ-treated or untreated PKC (2 × 10⁴ cells)). Viable HUVEC were evaluated by the water-soluble tetrazolium salt assay, after being incubated for up to 18 h, which measures the metabolic activity of viable cells by the formation of a formazan dye. We similarly evaluated the viability of HUVEC that had been incubated without either Aβ or PKC as a control experiment.

The effect of Aβ-treated PKC on the endothelial cell function was evaluated by treating PKC with 10 μM Aβ42 and then incubating with HUVEC in 100-mm dishes, using the procedure just described. Total RNA was isolated with an RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA) after 12 h of incubation. HUVEC were incubated without Aβ or PKC as a control sample, and total RNA was prepared. These RNA samples were subjected to reverse transcription, labeling, and hybridization to the GeneChip Human Gene 1.0 ST array (Affymetrix, Santa Clara, CA, USA). The array was scanned with a DNA microarray scanner (GeneChip™ System) according to the manufacturer’s instructions.

Data are expressed as the mean ± standard deviation (SD). We performed a statistical analysis by using two-way ANOVA and subsequent Tukey-Kramer test for multiple comparisons among several groups. Differences are considered significant at p < 0.05.

We first evaluated the influence of Aβ on the adhesion of RBC to HUVEC by using flow cytometry. HUVEC are larger than RBC, thereby showing greater forward and side scattering. It was therefore possible to define an RBC region and a HUVEC region in a flow cytometric plot (Fig. 1A). PKC were treated in the present study with and without Aβ, before incubating with HUVEC. The changes in cell numbers in the RBC and HUVEC regions were monitored in the subsequent flow cytometric analyses, and the adhesion ratio of RBC to HUVEC (the RBC/HUVEC ratio) was calculated. We found that treating PKC with Aβ, especially Aβ42, resulted in a marked increase in RBC/HUVEC ratio in a manner dependent on the Aβ dose (Fig. 1B). Two-way ANOVA revealed significant interaction between the treatment and time (p < 0.05). The data therefore clearly support a previous finding by Ravi et al., and it appears that Aβ actually promoted the adhesion of RBC to endothelial cells.

As blood flows through the circulation system, RBC binding to the vasculature is normally minimal, even in the microcirculation. RBC therefore do not generally adhere to endothelial cells, as we observed following the incubation of native PKC (Aβ-untreated PKC) with HUVEC (Fig. 1). However, it is conceivable that once RBC are modified by Aβ, such RBC readily bind to the endothelial vasculature, which could slow the blood flow and reduce the oxygen delivery. Since Aβ reportedly adheres to complement receptor 1 on erythrocytes, this mechanism may be involved in the Aβ-mediated RBC adhesion to HUVEC. Further studies are needed to evaluate this mechanism. We found in this study that Aβ42 caused a higher RBC/HUVEC ratio than Aβ40 (Fig. 1) which may provide an explanation for the lower concentration of unbound Aβ42 relative to Aβ40 in human plasma.

Having verified the Aβ-induced adhesion of RBC to HUVEC (Fig. 1), we next investigated whether such binding disrupted the endothelial properties (i.e., the HUVEC viability). We found that treating PKC with Aβ, especially Aβ42, resulted in a decreased number of viable HUVEC (Fig. 2). The result of two-way ANOVA showed significant interaction between the treatment and time (p < 0.05). Such treatment did not induce apoptosis in HUVEC (data not shown), and control PKC had no effect on the HUVEC viability. These data therefore indicate that Aβ mediated the adhesion of RBC to endothelial cells, and elicited a cytotoxic effect on the endothelium.

Unlike RBC and HUVEC, neurons and their relationship with Aβ have been studied in depth. Some of those
studies have reported that Aβ showed neurotoxic activity by generating reactive oxygen species (ROS). The mechanism by which Aβ generated ROS is not fully understood, although one study implicated the involvement of the methionine residue at position 35 of Aβ. Considering these reports, one possibility for the observed Aβ-induced cytotoxicity (Fig. 2) is that Aβ caused binding of RBC to HUVEC, and induced ROS, thereby decreasing the HUVEC viability. A DNA microarray study was therefore conducted to assess this possibility as well as others. The DNA microarray analysis (Affymetrix GeneChip Human Gene 1.0 ST array, 28,869 genes) was performed by comparing two samples. The first sample used PKC treated with Aβ42 and subsequent incubation with HUVEC (Aβ42-PKC-HUVEC), while the second control sample used HUVEC incubated without Aβ or PKC. The majority of genes showed only small differences in expression, with ratios (Aβ42-PKC-HUVEC/control HUVEC) ranging from 1.2 to 0.8. However, there were 1,488 up-regulated genes (those with ratios greater than 1.2) and 2,115 down-regulated genes (those with ratios less than 0.8) in Aβ42-PKC-HUVEC. Of the up- and down-regulated genes, several were involved in oxidative and inflammatory stress (Table 1). It is possible when considering the relationship among oxidative stress, inflammation, and cytotoxicity that Aβ induced the adhesion of RBC to HUVEC, thereby causing oxidative stress and inflammatory responses in HUVEC which contributed to the decreased HUVEC viability (Fig. 2). We are presently investigating this possibility in greater detail.

We collected blood from a healthy donor to conduct the present study in a similar manner to that of our previous study. Since data would vary due to measurement errors and biological variation, we are going to prepare PKC from a number of healthy volunteers in the next study. Nevertheless, it is worth noting that preliminary experiments using blood obtained from several donors showed the same effect of Aβ on erythrocytes and the endothelium (data not shown).

In conclusion, numerous studies have shown the involvement of oxidative and inflammatory stress in the pathogenesis of AD; for instance, we have previously found a higher accumulation of phospholipid hydroperoxide (an oxidative stress marker) in RBC of AD patients. It is likely from the results of our previous work and the present study that oxidative and inflammatory stress can, at least in part, originate from Aβ-mediated RBC adhesion to the endothelium. Such damage would be likely to injure the endothelial vasculature, resulting in impaired oxygen delivery, and this could initiate the cascade leading to AD pathology.

We have previously reported as a protective strategy that Aβ-induced RBC damage and RBC phospholipid peroxidation could be reduced by the use of carotenoids, especially xanthophylls Xanthophylls may therefore reduce the risk of AD. These possibilities warrant further testing on animal models of AD with the prospect of developing xanthophylls for human therapy.

### Table 1. Effect of Aβ42-Treated PKC on HUVEC Expression Levels of Oxidative Stress and Inflammation Genes

<table>
<thead>
<tr>
<th>Trivial name</th>
<th>Gene name</th>
<th>Function</th>
<th>Expression ratio (Aβ42-PKC-HUVEC/ control HUVEC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIG2</td>
<td>hypoxia-inducible protein 2</td>
<td>Response to stress</td>
<td>1.3</td>
</tr>
<tr>
<td>OXR1</td>
<td>oxidation resistance 1</td>
<td>Response to oxidative stress</td>
<td>1.2</td>
</tr>
<tr>
<td>GPX2</td>
<td>glutathione peroxidase 2</td>
<td>Response to oxidative stress</td>
<td>0.8</td>
</tr>
<tr>
<td>MT3</td>
<td>metallothionein 3</td>
<td>Response to hypoxia</td>
<td>0.8</td>
</tr>
<tr>
<td>SAA1</td>
<td>serum amyloid A1</td>
<td>Response to inflammation</td>
<td>1.6</td>
</tr>
<tr>
<td>CCR1L1</td>
<td>chemokine (C-C motif) receptor-1</td>
<td>Response to inflammation</td>
<td>1.5</td>
</tr>
<tr>
<td>IFNG</td>
<td>interferon, gamma</td>
<td>Response to inflammation</td>
<td>1.4</td>
</tr>
<tr>
<td>CCR1</td>
<td>chemokine (C-C motif) receptor 1</td>
<td>Response to inflammation</td>
<td>1.3</td>
</tr>
<tr>
<td>CXCL16</td>
<td>chemokine (C-X-C motif) ligand 16</td>
<td>Response to inflammation</td>
<td>1.3</td>
</tr>
<tr>
<td>IL1R1</td>
<td>interleukin 1 receptor, type 1</td>
<td>Response to inflammation</td>
<td>1.3</td>
</tr>
<tr>
<td>PLA2G4C</td>
<td>phospholipase A2</td>
<td>Response to inflammation</td>
<td>1.3</td>
</tr>
<tr>
<td>IL1RAPL1</td>
<td>interleukin receptor accessory protein-like 1</td>
<td>Response to inflammation</td>
<td>1.3</td>
</tr>
<tr>
<td>IL12A</td>
<td>interleukin 12A</td>
<td>Response to inflammation</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Fig. 2.** Effect of Aβ-Treated or Untreated PKC on HUVEC Viability.

Black and dark-gray bars indicate the HUVEC viability when PKC were respectively treated with 1.0 and 10 μM Aβ, before being incubated with HUVEC. Light-gray bars indicate the HUVEC viability when PKC not treated with Aβ were incubated with HUVEC. White bars represent the viability of control HUVEC that were incubated with neither Aβ nor PKC. Data are expressed as the mean ± SD (n = 5). Means without a common letter differ, p < 0.05.
Acknowledgments

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References