主 論 文

Phagocytosis of advanced glycation end products (AGEs) in macrophages induces cell apoptosis

（マクロファージにおける終末糖化産物の取り込みが細胞アポトーシスを誘導する）

[Introduction]

Diabetes is a group of disorders characterized by high glucose levels that cause unique eye, kidney, and nerve complications and an increased risk for cardiovascular disease. There are 425 million people with diabetes in the World. There will be 629 million people with diabetes in the World in 2045. Diabetes is a life-threatening condition and the more serious is complication. Especially the cardiovascular disorders, diabetes dramatically increases the risk of various cardiovascular problems, including coronary artery disease with chest pain (angina), heart attack, stroke and narrowing of arteries (atherosclerosis).

Many studies reported that under high glucose condition, the chemical transformation of amine-containing molecules by reducing sugars—whether on proteins, lipids or nucleotides resulted in advanced glycation end products (AGEs) or Maillard products. The formation of AGEs is an irreversible process. AGEs can combine covalently to proteins and precipitate in human tissues and finally cause tissue damage, over accumulation, chronic inflammation and chronic degradation disease like atherosclerosis and Alzheimer's. Although there are varies of oral hypoglycemic agents and insulin injection could be used in the treatment of diabetes, the metabolism of AGEs in human bodies still attracts many researchers to study on the role of AGEs in different diabetic complications. The reduction of AGEs' accumulation or promotion of its clearance from human bodies is becoming more and more meaningful as a new treatment strategy of diabetic complication. In another hand, macrophages are cells of hematopoietic system that have a crucial role in protective immunity and homeostasis. As an important immune cells, macrophages not only ingest and destroy invading pathogens, but also are charged with clearing dead and dying host cells.

This study aimed to clarify the interaction between AGEs and macrophages through in vitro study. First we collected information about the effect of AGEs on macrophages and got a hypothesis that AGEs could be incorporated into macrophages. The uptake of AGEs may induce a series of changes of cells and finally will be cleared from human bodies.

The study is mainly with the following step: 1. Synthesis of AGE-1,AGE-2, AGE-3, AGE-4

and AGE-5; cell culture of macrophages J774.1 cell line. 2. Fluorescence detection of intracellular AGEs in in the absent or presence of TritonX-100 under different temperature. 3. Fluorescence detection of AGEs' subcellular localization in cytosolic area or cell membranes. 4. Detect the influencing factor of the uptake of AGEs into macrophages with the proteinase inhibitor and receptor blocker. 5. Detect the inflammatory cytokines release from macrophages after stimulated with AGEs. 6. Detection of cell apoptosis. 7. Detect the cell signal pathway of AGEs' incorporation into macrophages. 8. Look for an effective and nontoxic treatment strategy for inhibiting the over accumulation of AGEs in human bodies.

[Materials and Methods]

Reagents: AGEs were produced at our laboratory using BSA from Sigma (St. Louis, MO) as substrate proteins. The antibodies against AGEs were raised according the method of Morioka et al. [10] as mentioned below. The following reagents were purchased from commercial sources: RPMI- 1640 medium (Sigma); penicillin-streptomycin solution (Sigma); 200mM L-glutamine (Invitrogen, Carlsbad, CA); fetal bovine serum 16000 (Invitrogen); PBS, TBS and formaldehyde solution (Sigma); 10 mM Tris-buffered saline and formaldehyde solution (Katayama Chemical Indus- tries, Osaka, Japan); and Triton X-100 (Nacalai Tesque, Kyoto, Japan). Normal goat serum, normal rabbit IgG, AlexaFluor 488-labeled goat anti-rabbit IgG (H+L), and AlexaFluor 555 goat anti-mouse IgG (H+L) were obtained from Abcam (Cambridge, UK); rabbit-caspase-3 antibody from Cell Signaling (Danvers, MA); anti-NF-κB p65 anti- body (Rb pAb; 16502) from Abcam; antiphosphatidylserine antibody from Upstate Biotechnology (Lake Placid, NY); goat anti-rabbit IgG (HRP) and DAPI from Life Technology (Carlsbad, CA); and West Dura extended duration substrate, 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCF-DA), and nuclear and cytoplasmic extraction (NE-PER) reagents from Thermo Scientific (Waltham, MA).

Cells: The murine cell line J774.1 (BALB/c) was purchased from JCRB Cell Bank (Osaka, Japan). Cells were incubated at 37°C in RPMI1640 medium (containing 10% FBS, 200mM glutamine and 1% penicillin- streptomycin solution) in a 5% CO2 humidified atmosphere and passaged every 3-4 days. After three passages, the cell suspension (106 cells/ml) was divided into 0.1ml aliquots that were incubated for 2 hrs in wells of a 96-well plate; then the medium with FBS was removed. Cells with a new medium without FBS were co-incubated in the presence or absence of AGEs (Falcon 353219 microtiter plate).

Synthesis of AGEs: BSA was incubated under sterile conditions with 0.2 M glyceraldehyde

(Sigma-Aldrich) and glycol aldehyde (Sigma-Aldrich), respectively, in 0.2 M phosphate buffer for 7 days (pH 7.4 at 37°C). AGE-2 and AGE-3 correspond to Glycer-AGEs and Glycol-AGEs, respectively. Each of the AGEs-BSA was dialyzed at 4°C to remove free aldehyde. The degree of glycation on BSA (AGE-BSA fluorescence) was measured by spectrofluorometric detection at excitation of 370 nm and emission of 440 nm.

Anti-AGE-Specific Polyclonal Antibody: Antibodies were produced from Japanese white rabbits. Rabbits were immunized with 5 ml AGEs-BSA emulsified with the same volume of Freund's complete adjuvant (Wako). The rabbits received first booster injection after three weeks, followed by two additional booster injections every week with emulsified Freund's incomplete adjuvant. After the whole blood was collected from the immunized rabbits under anesthesia, the antisera titers were determined by immunoblot analysis. Immunoglobulin fractions was purified using MEP HyperCel (Pall, Port Washington, NY, USA). Anti-AGE-specific antibodies were purified by using each of AGEs-BSA immobilized on Sepharose beads (GE Healthcare, Buckinghamshire, UK).

Method: Immunofluorescence Detection of AGEs after Incorporation into J774.1 Cells. After incubation in the presence or absence of AGEs for 2 hrs, cells (105 cells/well) were fixed with 10% formaldehyde for 20min and then washed three times with PBS for 5min each time. When the cell membranes were disrupted, 10 min incubation with 0.2% Triton X-100/TBS was performed; then, 3% normal goat serum (0.1% T-TBS) was used as a blocking agent for immunofluorescence detection. After 30 min, the samples were incubated with rabbit polyclonal anti-AGE-2 or anti- AGE-3, or normal rabbit IgG (1: 250) at room temperature for 1 hr. After three washes in T-TBS, cells were incubated for 1 hr with a secondary antibody (AlexaFlour 488-labeled goat anti-rabbit IgG) at room temperature. The fluorescence intensity was determined by FlexStation 3 mutimode microplate reader within one hour after washing cells with 0.1% T- TBS three times. Before observing the cells, we changed the plate medium (0.1% T-TBS) into TBS (non-Triton X) solution. The cells were observed under fluorescent microscopy (Bio- zero BZ8000; Keyence, Osaka, Japan). The 3D pictures were taken by confocal microscopy (LSM 780 Zesis Jena, Germany). Intracellular ROS Assay. Intracellular ROS activity was detected by H2DCF-DA. We detected intracellular ROS after exposure of J774.1 cells to AGEs. The cells were plated at a concentration of 106 cells/ml (1 × 105 cells per well) into a 96-well plate. After stimulation by AGEs for 24hrs, the cells were washed by PBS three times and loaded with 5 μM H2DCFDA at room temperature in the

dark for 15min. The fluorescence was measured with excitation at 485 nm and emission at 510 nm. Fluorescence images of cells were taken by a BZ-X700 (Keyence, Osaka, Japan) fluorescence microscope. Western Blot Analysis of Proteins: To analyze the cellular proteins, 6-well plates were used, with each well containing 1 ml of cell suspension (5 × 106 cells/ml). AGE-2 and AGE- 3 were diluted with PBS to give the final concentration of 20 μg/ml or 100 μg/ml. Cells were pre-incubated at 37°C for 1 hr, then stimulated with AGEs for 0, 1, 2, 4, 6, 8, 12, and 24hrs. After obtaining cell pellets by centrifugation, cells were treated with SDS-PAGE sample buffer (10% SDS, 1% β-mercaptoethanol, 2% Tris-HCl (pH 6.8), 20% glycerin, 0.01% bromophenol blue, H2O). Proteins were separated by SDS-PAGE and electrophoretically blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA). After blocking with 1% BSA in PBS, the membranes were incubated with rabbit primary antibodies at 4°C overnight. After the incubation with goat anti-rabbit IgG (HRP), the protein bands were visualized by the luminal-based enhanced chemiluminescence (ECL) HRP substrate method (Thermo Fisher Scientific Inc.). An Image Quant LAS4000 system was used for detection. Phosphatidylserine Staining: After stimulation with 100 μl AGE-2 or AGE-3 for 12 hrs, J774.1 cells were fixed with 10% formaldehyde for 20min and then washed three times for 5 min each in PBS. The buffer 0.2% Triton X-100/ TBS could not be used because of the disruption of cell membrane. Blocking with 3% normal goat serum (0.1% T-TBS) for 30 min was performed before immunostaining. In these experiments, double immunostaining of cells with mouse anti-phosphatidylserine was performed. Alexa Fluor 555 goat anti-mouse IgG (H+L) and Alexa Fluor 488-labeled goat anti-rabbit IgG were used as secondary antibodies for double staining.

Statistical Analysis: Statistical significance was evaluated by ANOVA followed by Dunnett's test for multiple comparisons, or by Student's t-test for comparisons between two groups. P values less than 0.05 were considered significant.

[Results]

1. Uptake of AGEs in J774.1 Macrophages: Result shows the temperature-dependent uptake of AGE-2 and AGE-3 into J774.1 macrophages at 2 hrs after the start of incubation. The incorporation of AGE1, AGE4 and AGE5 were also performed in macrophages, however, the fluorescent intensity was not as higher as AGE-2 or AGE-3. At 4°C, low levels of AGE binding were observed on the cell surface, irrespective of the presence or absence of Triton X-100 during the detection of antigens by the primary antibody. At 37°C, cell membrane-associated staining was apparent in the absence of Triton X-100, especially in the

case of AGE- 3, whereas the immunoreactivities for both AGE-2 and AGE-3 were enhanced in the cell-membrane-associated area and the intracellular compartment in the presence of Triton X-100. Thus, the fluorescence intensities of AGEs in the presence of Triton X-100 were significantly higher than those in the absence of Triton X-100 at 37°C. No matter with or without the influence of Triton X-100, the incubation in 4 °C will suppress the fluorescent intensity of AGE-2 and AGE-3 in macrophages. Another finding was that with the stimulateon of AGE-2 and AGE-3 together did not influence there incorporation, respectively.

2. Time-Course and Concentration Dependency of the Intracellular Uptake of AGE-2 and AGE-3 and Their Subcellular Localization in Macrophages: We determined time-course changes in the uptake of AGE-2 and AGE-3 in macrophages. The uptake of AGE-2 and AGE-3 was time-dependent and the fluorescence intensities of AGE-2 and AGE-3 increased up to 30min. Although the fluorescence intensity of the cells was relatively constant thereafter, the distribution pattern of immunoreactivities was changed time-dependently and significantly with highly positive spots for both AGE-2 and AGE-3. Result also shows the concentration dependency of the uptake of AGE-2 and AGE-3 in macrophages at 30min. The concentration-dependency curves for AGE-2 and AGE-3 showed a saturable shape, suggesting the existence of upper limits of capacity of incorporation. With the microscope observation, it shows typical pictures of AGE-2 and AGE-3 immunoreactivities at 2 hrs after the start of incubation with AGEs. The incorporated immunoreactivities formed small granule-like structures inside the cells, and some of the granules appeared to be present inside the nuclei. The 3D pictures suggested that the interaction of AGEs with macrophages should include binding of AGEs to the cell surface, incorporation into the cytoplasm and nuclear compartments, and granule-like formation. Result shows the distribution of AGE-2 and AEG-3 in macrophages, with both cell membrane binding and cytoplasmic incorporation. Time course result shows the highest fluorescence was observed at 12hrs and after 24hors, the fluorescence have a trend of discretion. Over 48hrs, very weak fluorescence staining can be observed in the result pictures.

3. Effects of Inhibitors of Endocytosis on Incorporation of AGEs in Macrophages: To examine the mechanism of AGE uptake into macrophages, cells were preincubated with cytochalasin D, chlorpromazine, inhibitor of RAGE FPS-ZM1 for 1 hr before the start of AGE uptake. The presence of cytochalasin D, an inhibitor of actin polymerization, blocked

AGE-2 and AGE-3 uptake in macrophage. The inhibition by cytochalasin D was concentration-dependent and the inhibitory effect was significant at a lower concentration of 5 μg/ml. However, chlorpromazine, an inhibitor of clathrin-dependent endocytosis, did not produce significant effects on incorporation of AGEs up to the concentration of 20-μg/ ml. the addition of FPS-ZM1, an antagonist of RAGE, enhanced the incorporation of both AGE-2 and AGE-3 in J774.1 cells in a concentration- dependent manner. The enhancing effects of FPS-ZM1 reached to maximal at 100 nM.

4. Intracellular ROS Production during AGE Incorporation into Macrophages: Intracellular ROS Production during AGE Incorporation into Macrophages. ROS production in macrophages stimulated by AGEs was detected by DCF fluorescence after uptake of DCF-AM. Both AGE-2 and AGE-3 (100μg/ml) elicited ROS production strongly in some populations of cells, whereas the remainder of cells produced lower levels of ROS 24hrs after the start of stimulation. In the PBS and BSA control groups, there were no detectable levels of ROS in the cells. As the bright-field image shows, the total numbers of cells were reduced in the groups exposed to AGE- 2 and AGE-3. ROS production in macrophages stimulated by AGEs was detected by DCF fluorescence after uptake of DCF-AM. Both AGE-2 and AGE-3 (100μg/ml) elicited ROS production strongly in some populations of cells, whereas the remainder of cells produced lower levels of ROS 24hrs after the start of stimulation. In the PBS and BSA control groups, there were no detectable levels of ROS in the cells. As the bright-field image shows, the total numbers of cells were reduced in the groups exposed to AGE-2 and AGE-3.

5. Apoptosis of Macrophages after Uptake of AGE-2 and AGE-3: We next checked the apoptosis-inducing effects of AGEs because intracellular ROS production may be associated with the induction of apoptosis. Since the activation of caspase-3 is one of the markers of apoptosis, we determined the activated form of caspase-3 (cleaved caspase-3) on Western blotting. We stimulate the cells with AGE2 or AGE-3 in a concentration of 20μg/ml, 50μg/ml or 100μg/ml. Pro-longed incubation with AGE-2 and AGE-3 at 20μg/ml for 24 hrs significantly produced cleaved caspase-3. The cleaved caspase-3 in the AGE-2 group was faint compared with that in the AGE-3 group; however, the higher concentration of AGEs at 100 μg/ml clearly showed that cleavage of caspase-3 was enhanced compared with the control groups. The cleavage of caspase-3 bind was detected at 12 hrs at a concentration of 100μg/ml. This bind was much deeper and clearer than any other time and a lower concentration.

Incubation with AGE-2 and AGE-3 induced the expression of phosphatidylserine (PS) detected by annexin V binding on the cell surface 12 and 24hrs after the start of incubation. The PS-positive cells were precisely merged with the cells exhibiting high AGE uptake. These results showed that AGE-2 and AGE-3 induced cell apoptosis and that the ability of AGE-3 to induce cell apoptosis was stronger than that of AGE-2.

6. AGE-Induced 4-Hydroxynonenal (4-HNE) Production and Activation of the NF-κB Pathway in Macrophages: We determined the translocation of NF-κB p65 from the cytosolic to the nuclear compartment to evaluate NF-κB activation. Results show that most of NF-κB immunoreactivity in the control cells was located in the cytosolic compartment. However, the NF-κB p65 immunoreactivity was partially translocated into the nuclear compartment after stimulation with AGE-2 and AGE-3. These immunocytochemical findings were consistent with the results of Western blotting, which showed the nuclear translocation of NF-κB p65 by stimulation with AGE-2 and AGE-3. However, since AGEs were incorporated into macrophages in a time and dosage dependent manner, there is no translocation of NF-κB p65 under 20μg/ml of AGEs. Another result is using AGEs to stimulate macrophages and detect intracellular 4-NHE expressions. The cells stimulated with AGE-2 and AGE-3 also showed constant production of 4-HNE. This is consistent with ROS production during AGE uptake. And strong immunofluorescence intensity can be detected when the concentration of AGEs is up to 100μg/ml.

7. Same experiment was repeated with RAW cells. Another cell lines RAW 274 macrophages cell line was used to repeat all the same experiment above. As expected the incorporation experiment result was in consistent with the results by using j774.1 cell line.

[Discussion]

AGEs have been suggested to be involved in the pathogenesis of many kinds of diseases, including diabetic complications, atherosclerosis, nephropathy, and neuropathic pain. The nonenzymatic production of AGEs occurs in the presence of higher concentrations of glucose in individuals with diabetes. Moreover, oxidative stress probably facilitates the formation of AGE adducts via the modification of proteins. There may be a diverse range of AGE subspecies produced, among which AGE-2 and AGE-3 exhibit strong toxic effects on specific cells. Receptors for advanced glycation end product (RAGE), SRA, LOX-1, and CD36 are the candidate receptors believed to mediate the various effects of AGEs. Soluble RAGE

treatment can cancel the effect of AGEs and be used as a biomarker in RAGE-dependent inflammation. However, the mechanisms by which AGEs are cleared from the extracellular environment are inadequately understood. Therefore, we examined the fundamental uptake mechanism of AGEs into macrophages and its relevant effects on cell viability to better understand the fate of AGEs. The present study clearly showed that J774.1 macrophages had the ability to incorporate AGE-2 and AGE-3 in a temperature-dependent and saturable manner. Cytochalasin D inhibits clathrin-independent endocytosis through depolymerizing F-actin. Chlorpromazine translocates chathrin and adaptor protein-2 from the cell surface to intracellular endosomes and inhibits clathrin-mediated endocytosis. The finding that cytochalasin D but not chlorpromazine inhibited AGE-2/3 uptake into J774.1 cells strongly suggests that AGE-2 and AGE-3 were taken up into macrophages mainly through clathrin-independent endocytotic mechanism but not through a clathrin-mediated mechanism. FPS-ZM1 is a high-affinity RAGE-specific blocker. In fact, we demonstrated that FPS-ZM1 blocked the binding of AGE-2 to sRAGE using surface plasmon resonance. Surprisingly, FPS-ZM1 enhanced both AGE-2 and AGE-3 uptake into J774.1 cells. Since AGE-2 and AGE-3 has been demonstrated to be high- affinity agonists for RAGE, the effects of FPS-ZM1 on AGE-2/3 uptake strongly suggest the inhibitory regulation by RAGE signaling on AGEs uptake mechanism. Thus, the signaling through RAGE may inhibit the clearance of AGE-2 and AGE-3, leading to the sustained presence of extracellular AGEs.

[Conclusion]

In conclusion, the present study clearly showed that macrophages incorporated toxic AGEs, AGE-2 and AGE-3, by a RAGE- or clathrin-independent endocytosis process. Stimulate macrophages with AGE-2 and AGE-3 did not influence their incorporation respectively. With additional stimulation of AGE-1, AGE-4 or AGE-5 also have no influence effect of their incorporation, respectively. Uptake of AGE-2 or AGE-3 into cells was in a time and dosage dependent manner and may be associated with ROS production and NF-κB activation. Accumulation of AGE-2 and AGE-3 inside macrophage cells in turn appears to trigger the activation of caspase-3, leading to apoptosis. These processes might in part reflect the events that occur in atherosclerotic plaques, especially with regard to foam cell death.