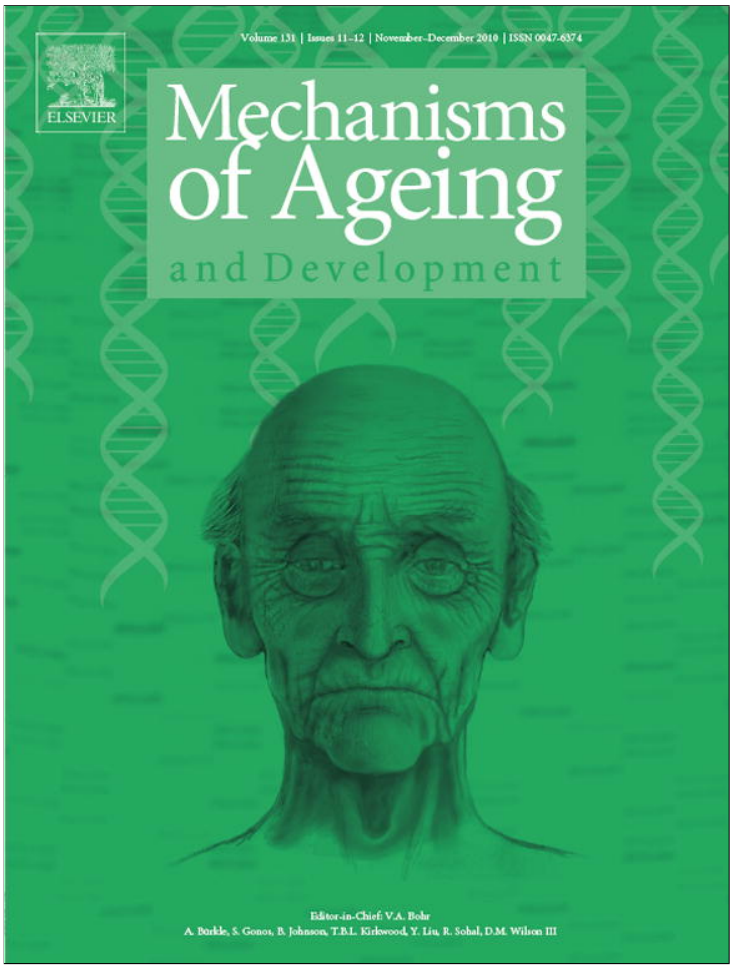

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Advanced glycation end products induce T cell apoptosis: Involvement of oxidative stress, caspase and the mitochondrial pathway

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ABSTRACT

Accumulation of advanced glycation end products (AGEs) is a hallmark in aged people. T cells play important roles in maintaining homeostasis of immune function. This study investigated the effects of AGEs-bovine serum albumin (AGEs) in human T cells. Incubation of Jurkat and several immortalized T cell lines with AGEs resulted in cell death dose-dependently. AGEs-induced cell death was partially but significantly blocked by neutralizing antibodies recognizing receptor of AGEs. In addition to detecting DNA nick, simultaneous stainings of annexin V with 7-amino-actinomycin D further confirmed the apoptotic nature of cell death. AGEs also caused apoptosis in purified T cells. Although AGEs-induced apoptosis could be blocked by the pan-caspase inhibitor, Ala-Asp-fluomethyl ketone (Z-VAD-fmk), there was no activation of caspase-3, -5, -8 and -9. AGEs caused mitochondrial outer membrane permeabilization and this process was prevented by an antioxidant or Z-VAD-fmk. Furthermore, AGEs treatment led to translocation of apoptosis inducing factor (AIF) from the mitochondria into the nucleus. Altogether, this report demonstrated that AGEs induced T cell apoptosis in an oxidative stress-associated and caspase-dependent manner with involvement of the mitochondrial pathway. It is likely that AGEs-induced T cell apoptosis may play a role in T cell homeostasis in ageing.

1. Introduction

Advanced glycation end products (AGEs) are non-enzymatic glycation products of proteins during the Maillard reaction and as a result accumulate along with the physiological ageing process. The accumulation process of AGEs is irreversible and the rate of their accumulation in different tissues depends on several factors, including longevity of the modified proteins, oxidative processes, availability of metal ions and redox balances (Glenn and Stitt, 2009). In addition to playing critical roles in the pathogenesis of diabetes, atherosclerosis, and renal disorders, AGEs accumulation is also pathogenic in many tissues of aged people (Cardenas-Leon et al., 2009). One of the pathogenic mechanisms from AGE accumulation is through inducing cellular apoptosis. Such a pro-apoptotic effect of AGEs has been demonstrated in several cultured tissue cells such as retinal pericytes, corneal endothelial cells, neuronal cells, and renal mesangial cells (Denis et al., 2002; Kaji et al., 2003; Kasper et al., 2000). Molecular analysis of the pro-apoptotic mechanisms of AGEs reveals involvement of induction of the mitogen-activated protein (MAP) kinase pathway, the caspase-dependent pathway, the mitochondrial pathway as well as the generation of reactive oxygen species (ROS) (Alikhani et al., 2007; Chen et al., 2006; Li et al., 2007). Binding of AGEs to its receptors also activates nuclear factor-kappa B (NF-κB) and leads to the generation of pro-inflammatory cytokines, vasoconstriction, increase in the procoagulant state, and enhances expression of adhesion molecules (Nienhuis et al., 2009; Soro-Paavonen et al., 2008). Given these promising reports in examining the significance of AGEs in many tissue cells, the effects and mechanisms of AGEs in human T cells remain largely unknown.

The study examining the immune system in the elderly, the SENIEUR-Protocol, reveals that the number of lymphocytes decreases, specifically the number of $CD3^+$ T cells (Ibs and Rink, 2001; Rink and Seyfarth, 1997). A large population study comprising of 513 elderly people indicates that along with increasing age, there is a significant decrease of $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ cells, and total lymphocytes, especially in those older

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than 80 years old (Huppert et al., 1998). Similar conclusion was also reached recently by other researchers (Czesnikiewicz-Guzik et al., 2008). Furthermore, an analysis in determining the ranges of peripheral blood lymphocyte subsets in Chinese healthy adults shows the inverse correlation between ageing and the $CD3^+$ T cell population (Jiao et al., 2009). Different from these reports, a study examining 29 aged people and 21 healthy young control subjects does not reveal any difference in total numbers and subsets of lymphocytes (Carson et al., 2000). In addition, there are several other reports indicating that T cell numbers remain constant with ageing although there is a considerable decrease in CD4- and CD8-mediated responses [reviewed in Grubeck-Loebenstein and Wick, 2002]. The inconsistent results obtained may be due to several factors, including the variation in defining "elderly persons", varying analytical methods used, numbers of patients included for analysis and difficulties in controlling for concurrent illness or comorbidity of the examined populations.

Given the importance of T cells in fighting microbial infections, neurodegeneration, and cancer in aged people, we examined how the accumulation of AGEs may affect T cells. The work in this report demonstrated that AGEs treatment resulted in T cell apoptosis and the effects were likely to involve oxidative stress, caspase and the mitochondrial pathways; however, several caspases, including caspase-3, -5, -8 and -9 appeared not to participate in this process. The *in vitro* findings in this report suggested that AGEs-induced T cell apoptosis may play a role in T cell homeostasis in ageing.

2. Materials and methods

2.1. AGEs preparation

Glycoaldehyde-modified albumin, used as the source of AGEs in this study, was prepared as previously described with minor modification (Huang et al., 2009). In brief, crude AGEs were prepared by incubating 10 mg/ml of bovine serum albumin (BSA) (USB Corporation, Cleveland, OH, USA) in phosphate-buffered saline (PBS) containing 33 mM glycoaldehyde, 0.1% sodium azide, and 1 mM phenylmethylsulfonyl fluoride (PMSF) at 37 °C for 7 days. After dialysis against PBS, the AGEs preparation was filtered and stored at −80 °C until use. The following concentrations of AGEs: 400, 200, 100, and 50 μg/ml were examined in this study. By Western blot analysis, we observed that the preparation contained at least one of the major components of AGEs N^{ε}-(carboxy-methyl) lysine (CML) (Supplementary Fig. 1).

2.2. Preparation of peripheral blood T cells and culture of immortalized T cell lines

Jurkat T cells and other immortalized T cell lines, MOLT-4, CEM and SUP-T1, were purchased from American Type Culture Collection (ATCC) and grown in a RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 1000 U/ml of penicillin–streptomycin (Life Technologies, Gaithersburg, MD, USA). According to the catalogue, SUP-T1 cell line was derived from tumor cells collected from the malignant pleural effusion of an 8-year-old child with T lymphoblastic leukemia. MOLT-4 cell line was established from cells taken from a 19-year-old male patient of acute lymphoblastic leukemia in relapse. The patient had received prior multidrug chemotherapy and has a G → A mutation at codon 248 of the p53 gene. CEM (CCRF-CEM) cell line was established from peripheral blood T lymphoblast taken from a 4-year-old female patient with acute lymphoblastic leukemia. These T cell lines express multiple T lineage markers. The preparation of human peripheral blood T cells has been described in detail in our previous report (Ho et al., 2004). In brief, buffy coat from blood bank was mixed with Ficoll-Hypaque, after centrifugation, the layer of mononuclear cells was collected. After lysis of red blood cells, the peripheral blood mononuclear cells were laid on Petri dishes to remove adherent cells and then incubated with antibodies, including L243 (anti-DR; ATCC), OKM1 (anti-CD11b; ATCC), and LM2 (anti-Mac1; ATCC) for 30 min at 4 °C. The cells were then washed with medium containing 0.1% FBS and incubated with magnetic beads conjugated with goat anti-mouse IgG (Dynabeads, Invitrogen, USA). The antibody-stained cells were then removed with a magnet. Following a repeat of the above procedures, the T cells were obtained with purity more than 98% as determined by the percentage of $CD3^+$ cells in flow cytometry (Beckton Dickinson).

2.3. Measurement of non-specific cytotoxicity

Several assays were used to examine the potential cytotoxicity of AGEs. The release of lactate dehydrogenase (LDH), as an indicator of damage to the plasma membrane and cell death, was measured according to the manufacturer's instructions (Roche, Indianapolis, IN, USA). The percent cytotoxicity was calculated as ([sample value − medium control]/[high control − medium control]) × 100, where the sample values were the averages of the absorbance values from triplicates of BSA- or AGEs-treated cell culture supernatants after the subtraction of the absorbance values from the background control. Similarly, the average absorbance values of untreated cell culture supernatants, used as the medium control, were calculated. Equal amount of cells treated with 1% Triton X-100 was used as the high control. The trypan blue exclusion and tetrazolium salt 3-[4,5-dimethylth-iazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assays were performed as previously described (Lai et al., 1999, 2001).

2.4. Measurement of cellular apoptosis by flow cytometry

After washing with cold PBS, cells treated with different concentrations of AGEs and untreated cells were pelleted and resuspended in a binding buffer containing HEPES-buffered PBS supplemented with 2.5 mmol/l $CaCl_2$. Then 10 μl annexin V (10 μg/ml) and 7-AAD (10 μg/ml) were added to each sample, and the mixture was incubated for 15 min at room temperature. After washing, the cells were analyzed by flow cytometry (Becton Dickinson). The stainings of 7-AAD and annexin V were used to distinguish living cells (7-AAD-/annexin V−), early apoptotic (7-AAD-/annexin V+), late apoptotic (7-AAD+/annexin V+) and necrotic cells (7-AAD+/annexin V−). When PI staining was measured alone, the cells were pelleted and resuspended in 1.5 ml hypotonic fluorochrome solution containing 50 μg/ml PI, 0.1% sodium citrate, and 0.1% Triton X-100 (Sigma). The cells were left overnight at 4 °C in the dark. While the cells were not permeabilized for simultaneous staining, Triton X-100 was added to permeabilize the cells stained only with PI. The PI fluorescence intensity was then measured with flow cytometry, and the subdiploid DNA content was analyzed with the CellQuest program (Becton Dickinson).

2.5. Measurement of DNA fragmentation using the TUNEL assay

To evaluate DNA fragmentation using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL), 200 μl of 2% fixative solution was added to the cultured T cells and they were oscillated for 1 h. The cells were washed with 1 ml PBS and centrifuged at 1000 × *g* for 10 min at 4 °C. After that, 100 μl of permeabilization solution was added to the cell culture for 2 min in the ice. After adding 50 μl of the TUNEL reaction mixture to the cells for 1 h at 37 °C in the dark, the cells were washed twice. Finally, the samples were mixed in 550 μl PBS and evaluated by flow cytometry under an excitation wavelength of 488 nm and a detection wavelength of 518 nm. Both geometric means of fluorescence intensity and percentages of TUNEL+ cells were individually used to evaluate the strength of cellular apoptosis. In addition, we placed 50 μl of the sample on a slide to capture images using a microscope.

2.6. Caspase activity by flow cytometry

This procedure was performed according to the manufacturer's instructions (BD Pharmigen, CA, USA). In brief, the untreated and treated cells were pelleted after washing and were fixed overnight with 1% paraformaldehyde. After washing with cold PBS, the cells were permeabilized using 0.25% saponin. A phycoerythrin-conjugated anti-caspase monoclonal antibody was added and the cells were incubated for 30 min at 4 °C in the dark. After washing, the active caspase product was analyzed using flow cytometry (Becton Dickinson). In separate reactions, 100 μM of the pan-caspase inhibitor Ala-Asp-fluomethyl ketone (Z-VAD-fmk) (Calbiochem, Cat:627610) was added to the T cells before treatment with AGEs for the evaluation of caspase-associated cellular apoptosis.

2.7. Western blotting

ECL Western blotting (Amersham-Pharmacia) was performed as described (Ho et al., 2005). Briefly, equal amounts of whole cellular extracts were analyzed on 10% SDS-PAGE and transferred to the nitrocellulose filter. For immunoblotting, the nitrocellulose filter was incubated with TBS-T containing 5% non-fat milk for 2 h and then blotted with antibody against specific proteins for another 2 h at room temperature. After washing with milk buffer, the filter was incubated with rabbit anti-goat IgG or goat anti-rabbit IgG conjugated to horseradish peroxidase at a concentration of 1:5000 for 30 min. The filter was then incubated with the substrate and exposed to X-ray film (Kodak).

2.8. Evaluation of ROS accumulation and mitochondrial function

For the detection of intracellular ROS in cultured T cells, 2.5 μM ROS staining agent (5-(6)-chloromethyl-2,7′-dichlorodihydrofluorescein diacetate) was incubated with cultured T cells for 30 min at 37 °C. Intracellular oxidative stress was determined by using flow cytometry under an excitation wavelength of 488 nm and a detection wavelength of 518 nm. For the evaluation of mitochondrial membrane potential, 35 nM of a mitochondrial staining agent (DiOC6; Molecular Probes) was added to the cultured T cells for 30 min at 37 °C, and analyzed by flow cytometry. In separate experiments, 10 μM of an antioxidant agent, N-acetyl-cysteine (NAC), was added to the T cells before the addition of AGEs to evaluate the accumulation of ROS.

2.9. Detection of apoptosis inducing factor (AIF) migration

After treatment, cell suspensions were cytospined onto a polylysine-coated slides and the cells were fixed by immersing in 4% paraformaldehyde for 20 min. Cells were permeablized by adding 1% v/v Triton X-100 for 20 min and then followed by adding 1% BSA to block non-specific binding. Subsequently, mouse anti-human AIF monoclonal antibodies (Santa Cruz) was used to stain the cells and PE-conjugated anti-mouse IgG (Chemicon) was used as the secondary antibody. After staining the nucleus by DAPI, the slides were visualized under a fluorescence microscope.

2.10. Statistical analysis

When necessary, the results were expressed as the mean ±standard deviation (SD). The one-way ANOVA was used to analyze the data; $P < 0.05$ was considered significant.

3. Results

3.1. AGEs-induced T cell death

To determine whether AGEs affected T cells, Jurkat T cells were cultured with different concentrations of AGEs for 5 days, and the cells were analyzed using trypan blue exclusion, MTT, and LDH assays. AGEs treatment reduced T cell counts in trypan blue exclusion assays (Fig. 1A) and increased T cell death in MTT assays (Fig. 1B). The LDH assays detected the amount of NADH released from dead cells and the results indicated that higher doses of AGEs induced significant cytotoxicity of T cells (Fig. 1C). When Jurkat T cells were pre-incubated with neutralizing antibodies recognizing receptor of AGE (RAGE) before treatment with AGEs, we observed that AGEs-mediated T cell death was partially and yet significantly blocked (Fig. 1D). In addition, we showed that incubation with AGEs, there was no detectably increased expression of RAGE (Supplementary Fig. 2). In a side-by-side comparison with Jurkat T cells, three other immortalized T cell lines, MOLT-4, CEM and SUP T-1, were also susceptible to AGEs-induced cell death with SUP T-1 being the most sensitive one among them (Fig. 2). Regarding SUP T-1 cell line, according to the report (Smith et al., 1984), there are two noticeable characters of this cell line. First, SUP T-1 cell line did not form rosettes with sheep RBCs. Second, it lacked CALLA (common acute lymphoblastic leukemia antigen), an antigen present on 21% of the patient's malignant effusion. Whether these two characters might result in increased sensitivity to AGEs-induced apoptosis was not clear. We also found that there was no difference in RAGE expression between Jurkat and SUP T-1 cells (data not shown). Similar to that in Jurkat T cells, AGEs-induced death in SUP T-1 cells was also partially prevented by anti-RAGE antibody treatment (data not shown). Altogether, these results demonstrated that AGEs-induced cell death was a common finding in immortalized T cells and the effect could be partially and yet significantly blocked by neutralizing RAGE.

3.2. AGEs-induced T cell apoptosis could be demonstrated by the TUNEL assay and the stainings of annexin V-7-AAD

PI staining of DNA was used to evaluate whether AGEs induced T cell death by disrupting the cell cycle. We observed that T cell death was not caused by altering the cell cycle of T cells (Supplementary Fig. 3A and B). In order to determine the mechanism by which AGEs induced T cell death, we used the TUNEL assays to stain nuclei containing fragmented DNA. Under microscopic examination, AGEs treatment increased cell number containing DNA nick in a dose-dependent manner (Fig. 3A). By flow cytometry analysis, the increase of TUNEL intensity with statistical significance after AGEs stimulation was observed (Fig. 3B and C). The results indicated that AGEs induced T cell death via apoptosis.

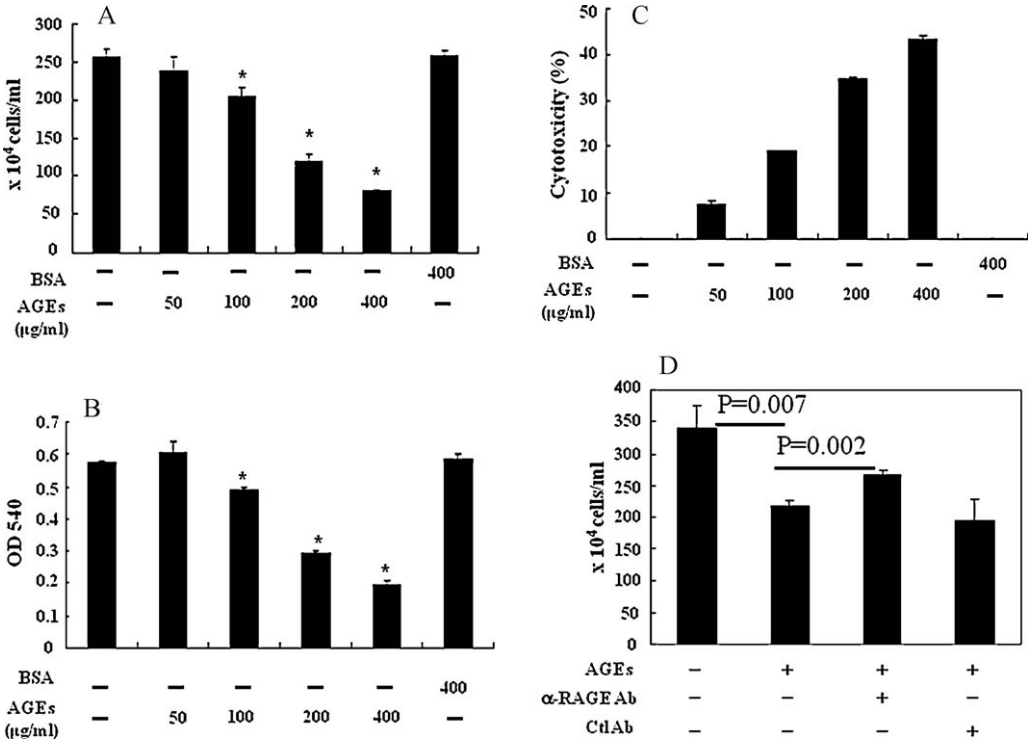

Fig. 1. AGEs induced T cell death that could be partially and yet significantly blocked by neutralizing RAGE. Jurkat T cell (10^5 cells/ml) were cultured with different concentrations of AGEs (0, 50, 100, 200 and 400 μg/ml) for 5 days, several approaches, including trypan blue exclusion, MTT, and LDH assays were used to examine the potential cytotoxicity of AGEs. (A) The trypan blue exclusion assay showed a significant reduction in T cell counts under different concentrations of AGEs treatment. (B) The MTT assay showed a significant reduction of viable T cells in a dose-dependent manner. (C) The LDH assay indicated that escalated concentrations of AGEs increased T cell cytotoxicity. (D) Before treatment with AGEs, Jurkat T cells were pre-treated with 80 μg/ml neutralizing antibodies recognizing RAGE (α-RAGE) or isotype-matched control antibodies (Ctl) for 2 h. The results suggested that blocking AGEs-RAGE interaction partially and yet significantly prevented AGEs-mediated death in Jurkat T cells. $^{\#}P < 0.05$ compared to control BSA. At least three independent experiments for each condition were performed.

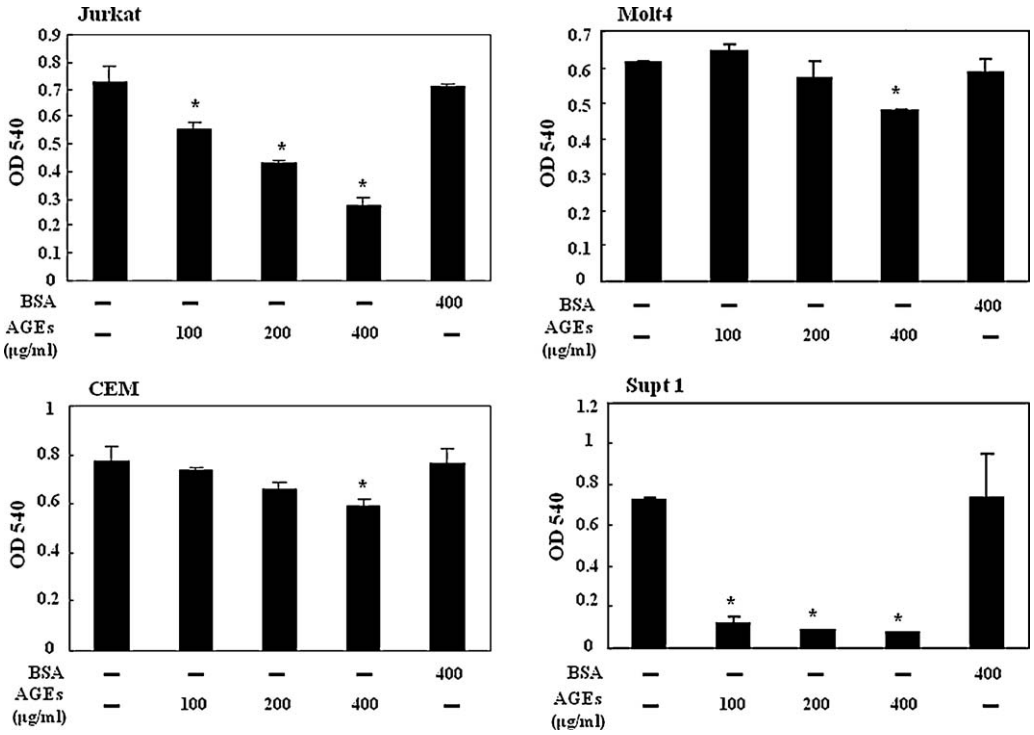

Fig. 2. AGEs induced cell death in four immortalized T cell lines. As a side-by-side comparison, similar to Fig. 1, in MTT assays, treatment with different concentrations of AGEs resulted in cell death in Jurkat, MOLT-4, CEM and SUP-T1 immortalized T cell lines. The results suggested that AGEs-mediated T cell death was a common phenomenon and could be generally observed in different T cell lines. At least three independent experiments for each condition were performed.

In order to confirm that AGEs-induced apoptosis could also be observed in primary T cells, TUNEL assays were applied to test this possibility. As shown in Fig. 4A and B, the results examining 4 different donors T cells revealed that AGEs induced apoptosis in purified human peripheral blood T cells. Moreover, the AGEs-mediated apoptotic mechanisms were further examined by using markers distinguishing different phases of apoptosis from necrosis. The flow cytometry approach in staining cells with annxin V and 7-AAD to distinguish living cells (7-AAD-/annexin V−), early apoptotic (7-AAD-/annexin V+) and late apoptotic cells (7-AAD+/annexin V+) from necrotic cells (7-AAD+/annexin V−) further confirmed that AGEs-induced T cell death was mediated through apoptotic mechanisms (Fig. 5). The results shown also indicated that the AGEs-induced T cell apoptosis began to be detectable within 2 days after treatment.

3.3. AGEs-induced T cell apoptosis is through a caspase-dependent but caspase-3, -5, -8, and -9-independent mechanism

Caspase enzymes are known to be involved in the signaling pathway of apoptosis and caspase-3 is an important effector enzyme in the caspase-dependent apoptotic pathway. To analyze the activation of caspase-3 in T cells, we measured the intracellular protein levels of active caspase-3 with flow cytometry. As shown in Fig. 6A, AGEs did not induce the activation of caspase-3. However, the pre-treatment with the pan-caspase inhibitor Z-VAD-fmk for 3 h partially and yet significantly blocked AGEs-induced nicking of DNA (Fig. 6B and C). It suggested that AGEs-induced T cell apoptosis was mediated through a caspase-dependent and caspase-3-independent signaling pathway. Further analysis with Western blot to determine whether other caspases might possibly be involved in AGEs-mediated T cell apoptosis showed that neither caspase-5, caspase-8 nor caspase-9 participated in this event (Fig. 6D). Because caspase-5 was not activated by cycloheximide treatment in Western blotting, an alternative approach to evaluate the role of caspase-5 was chosen. By using caspase activity assay kits detecting the activation of caspases, the results confirmed that caspase-3, -5, -8 and -9 were not induced by AGEs in contrast to the cycloheximide treatment (Supplementary Fig. 4). As shown in Supplementary Fig. 5A, similar to AGEs, incubation of Jurkat T cells with a pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α), took 3–5 days to induce significant T cell apoptosis. However, different from AGEs, TNF-α treatment effectively induced the activation of several caspases (Supplementary Fig. 5B).

3.4. AGEs-induced mitochondrial outer membrane permeabilization (MOMP) via ROS generation that was also inhibited by Z-VAD-fmk

Subsequently we examined whether AGEs-induced apoptosis in Jurkat T cells involved production of ROS. The results revealed that a significant accumulation of ROS was observed after T cells were treated with AGEs (Fig. 7A). Because MOMP plays an important role in the progression of cell death and can trigger caspase-dependent and caspase-independent apoptosis, it was evaluated in AGEs-treated T cells. We used DiOC6 staining to detect the function of the mitochondrial membrane. Flow cytometry analysis revealed a dose-dependent increase of DiOC6-negative population after AGEs treatment (Fig. 7B). This finding indicated that MOMP was triggered by AGEs. The addition of an antioxidant N-acetyl-cysteine (NAC) effectively blocked AGEs-induced loss of function of the mitochondrial membrane (Fig. 7C). It suggested that the pathological accumulation of ROS was a key trigger for MOMP. Moreover, the pre-treatment with 100 μM Z-VAD-fmk also successfully blocked AGEs-induced MOMP (Fig. 7D). It suggested that certain Z-VAD-fmk-susceptible caspase might play upstream of the event of MOMP induced by AGEs.

3.5. Involvement of oxidative stress in AGEs-induced T cell apoptosis

In addition to regulating AGEs-induced loss of mitochondrial potential, the role of oxidative stress in AGEs-mediated T cell

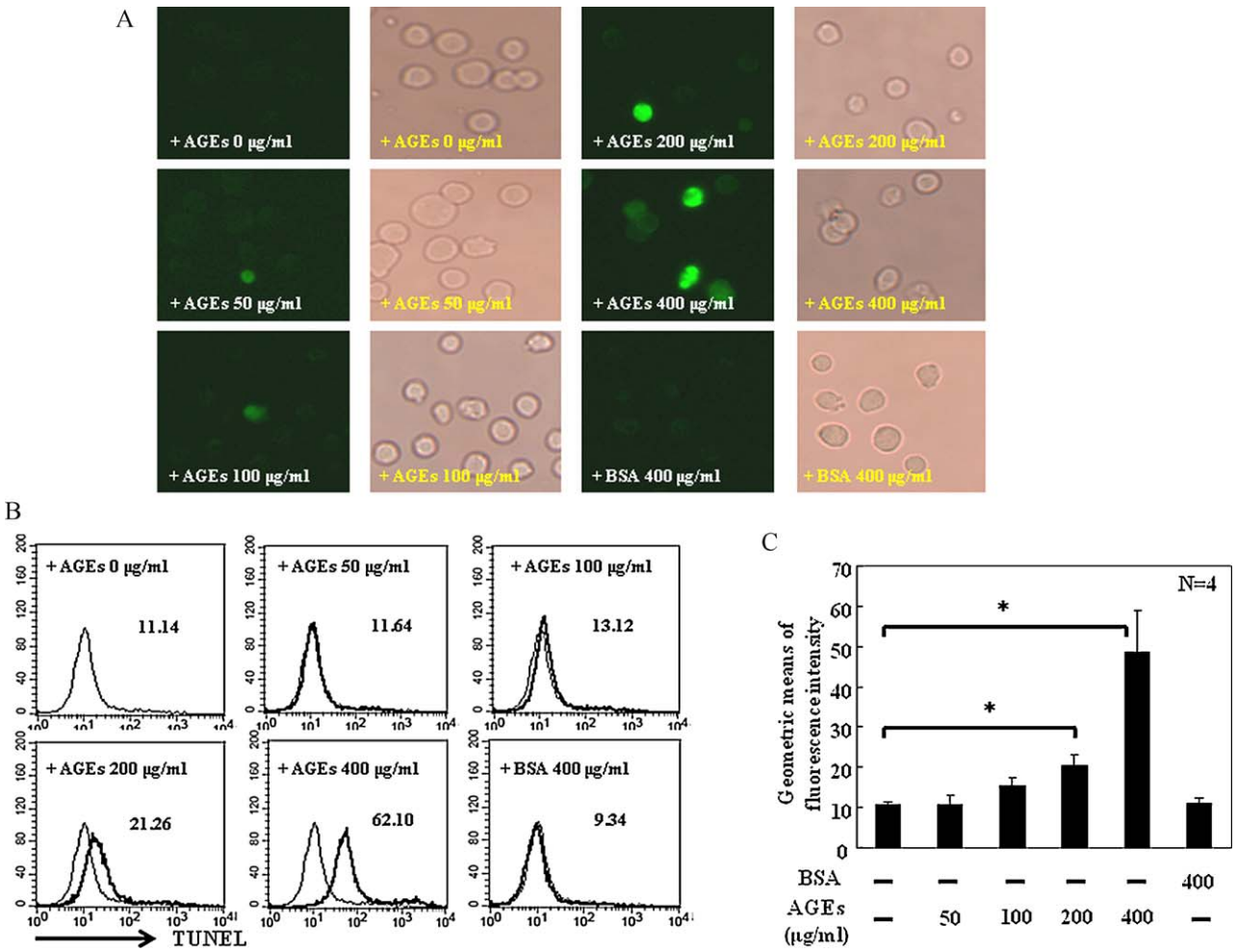

Fig. 3. In TUNEL assays, significant DNA nicking occurred during the progression of AGEs-induced T cell apoptosis. (A) The positivity and intensity of immunofluorescence in the AGEs-treated groups were higher than that in the control group. (B) Flow cytometry analysis revealed the same conclusion that increasing intensity of TUNEL was observed only in the groups treated with AGEs. The results from four independent experiments were pooled together and analyzed (C). Values shown are geometric means of fluorescence intensity. $^{*}P < 0.05$.

apoptosis was examined. Because, we could not detect increase of caspase activity in AGEs-treated T cells, TUNEL assays were chosen to address this issue. The results showed that the antioxidant NAC significantly prevented AGEs-induced T cell apoptosis (Fig. 8A and B). It suggested that oxidative stress directly participated in AGEs-induced T cell apoptosis.

3.6. Migration of mitochondrial AIF into the nucleus in AGEs-induced T cell apoptosis

To examine whether the mechanism of AGEs-induced T cell apoptosis was associated with the migration of AIF, we applied immunocytochemistry analysis with monoclonal antibodies recognizing AIF during the progression of T cell apoptosis. After T cells were cultured with AGEs for 5 days, the immunofluorescence staining showed significant AIF migration from the mitochondria to the nucleus (Fig. 9). The results suggested that AIF might be an important mitochondrial protein released as a result of MOMP and could promote AGEs-induced T cell apoptosis.

4. Discussion

AGEs derived from an array of precursor molecules preserve great heterogeneity of chemical structures. Identified AGEs *in vivo* include CML, crossline, pentosidine, glucosepane furoyl-furanyl imidazole, hydroimidazolone, argpyrimidine, 1-alkyl-2-formyl-3,4-glycosyl-pyrrole, glyoxal lysine dimer, and methylglyoxal lysine dimer and many of these accumulate along the ageing process (Glenn and Stitt, 2009; Thorpe and Baynes, 2003). The serum concentration of pentosidine for the group aged 80–93 years can be three times higher than that for the group aged 20–29 years (Takahashi et al., 2000; Yoshihara et al., 1998). As reported by Xu et al. (Xu et al., 2003), there is a good correlation between the serum level of CML and the serum level of AGEs suggesting the sharing of similar biological process in the formation of different AGEs species. As calculated, the concentrations of 50, 100, and 200 μg/ml AGEs are comparable to 7.5, 15, and 30 U/ml CML, respectively (Xu et al., 2003). The high measurable concentrations of CML are around 25–30 U/ml in aged people (aged >60 years) (Uribarri et al., 2007). In mice fed with regular diet, the concentrations of CML measured by monoclonal antibodies recognizing CML-like epitope can reach up to 60 U/ml (Cai et al., 2008). Accordingly, the ranges of AGEs concentrations used in the present study (50–400 μg/ml) should be close to the physiologically detectable serum concentrations of AGEs in aged people.

Within the examined concentrations of AGEs, we demonstrated that AGEs could cause cell death in several immortalized T cell lines and primary T cells. It suggested that the observed effects of AGEs in T cells were common phenomenon and were not only specific for Jurkat T cells. Investigation of the mechanisms by

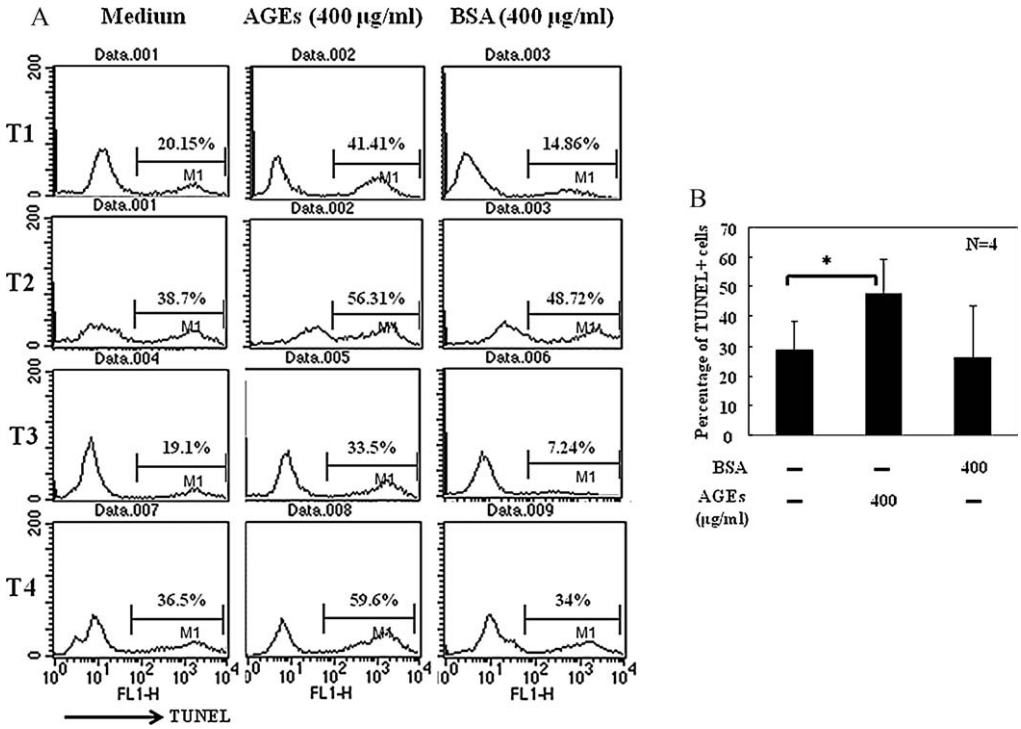

Fig. 4. Induction of apoptosis by AGEs was observed in primary T cells. (A) Primary T cells were purified from buffy coat of four different donors and the susceptibility of individually collected T cells to AGEs-induced apoptosis was examined by TUNEL. Similar to immortalized T cell lines, AGEs caused apoptosis in primary T cells. The results from four independent experiments showing percentage of TUNEL+ cells were pooled together and analyzed (B). T1, T2, T3 and T4: T cells from donors 1, 2, 3 and 4, respectively.

staining PI, annexin V and 7-AAD and by measuring DNA nick confirmed that AGEs induced T cell death through apoptosis rather than through necrosis. Although subject to disputation whether accumulated T cells in aged people are more susceptible or more resistant to apoptosis (Bryl et al., 2001; Herndon et al., 1997; Phelouzat et al., 1996; Phelouzat et al., 1997; Spaulding et al., 1999; Spaulding et al., 1997), the results in this report seemed to indicate that under the environment of high concentrations of AGEs, T cells were prone to undergo apoptosis. It is noteworthy that the different conclusions obtained from these studies may suggest the complexity of examining T cells in aged and several factors such as the stimuli used, the phenotype of the T cells

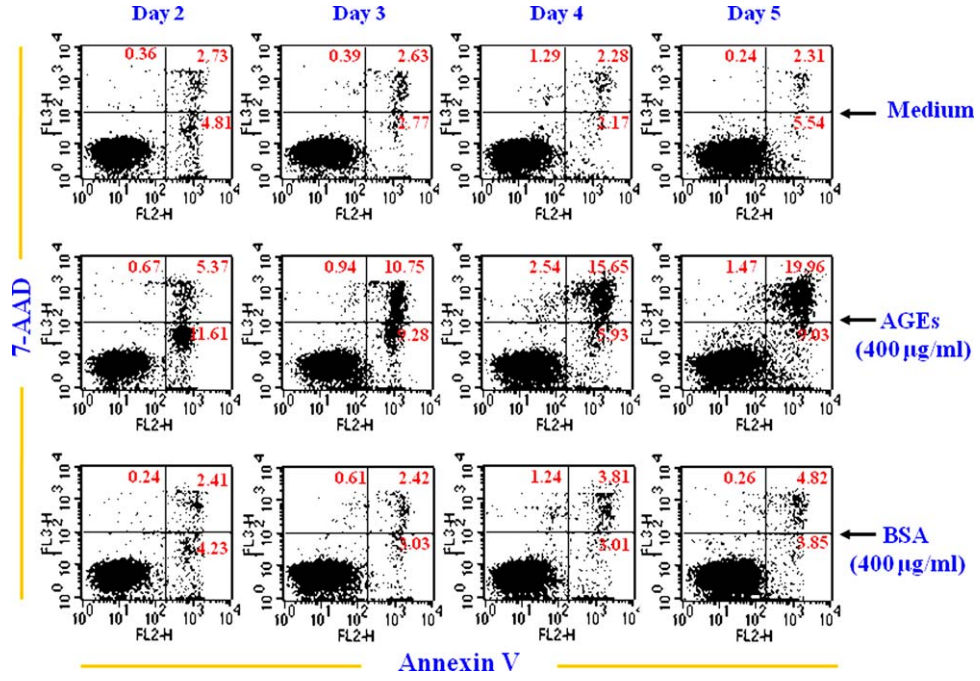

Fig. 5. AGEs induced T cell apoptosis as demonstrated by evaluating the stainings of annexin V and 7-AAD. Stainings with both annexin V and 7-AAD revealed gradually increased populations of annexin V+/7-AAD- and then annexin V+/7-AAD+; however, no increased population of annexin V-/7-AAD+ cells after AGEs treatment was observed. These findings concluded that AGEs induced T cell apoptosis rather than necrosis. At least three independent experiments for each condition were performed.

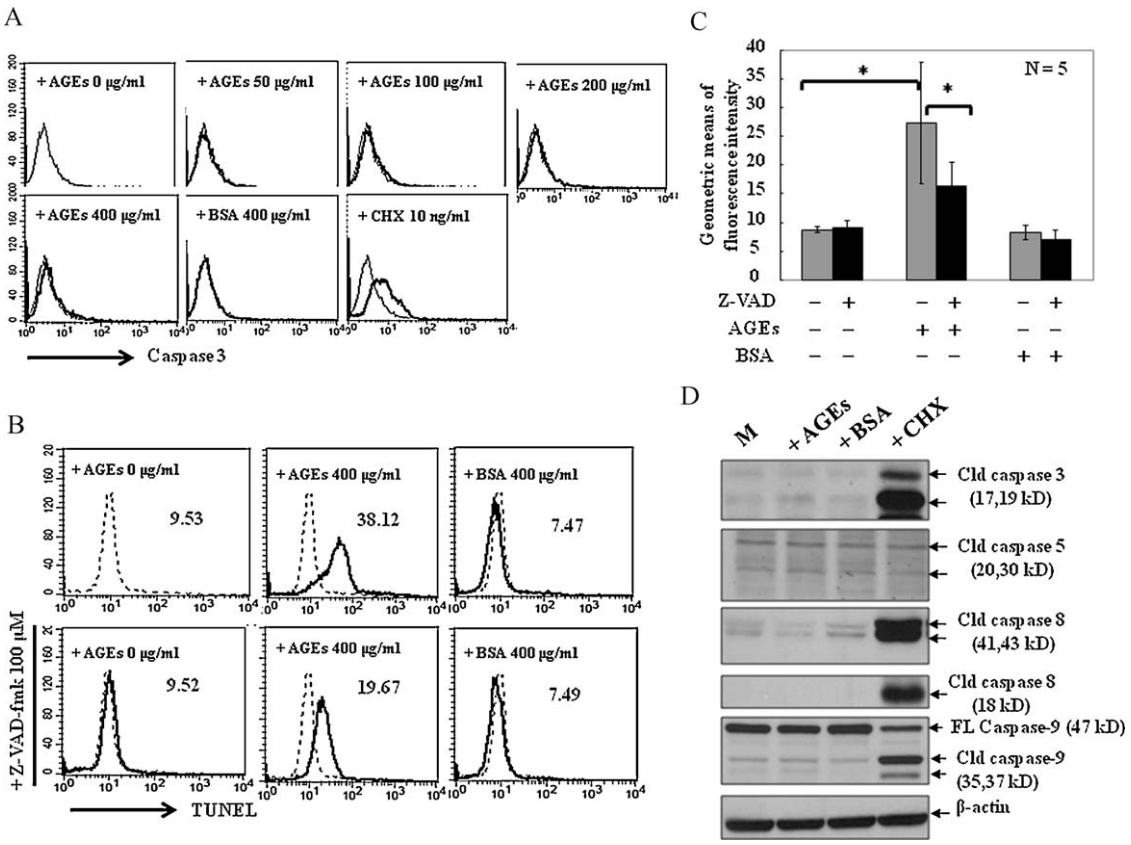

Fig. 6. AGEs treatment did not induce caspases activity; however, the pan-caspase inhibitor Z-VAD-fmk partially and yet significantly blocked AGEs-induced DNA nick in Jurkat T cells. (A) By flow cytometry analysis, there was no significant increase of active caspase-3 level in Jurkat T cells treated with different concentrations of AGEs. As a positive control, cycloheximide (CHX) triggered significant caspase-3 activation. (B) In TUNEL assays, significant DNA nicking induced by AGEs treatment was partially and yet significantly blocked in the presence of Z-VAD-fmk. (C) Pooled results from 5 independent experiments were statistically analyzed. (D) In Western blot analysis, compared to CHX treatment, Jurkat T cells treated with AGEs did not activate caspase-3, -8 and -9. Using this approach, we could not detect the activation of caspase-5 by both stimuli (please refer to Supplementary Fig. 4). Cld: cleaved; FL: full length. At least three independent experiments for each condition were performed.

studied as well as the models examined can potentially be different and affect the outcomes (Hsu et al., 2005).

Incomplete blockade by neutralizing RAGE antibodies suggested that the effects from AGEs treatment might be mediated through many different pathways and RAGE participated in only part of the effects. Indeed, AGEs can elicit the effects through interacting with numerous receptors and binding proteins which are either inflammatory receptors such as RAGE and AGE-R2 or clearance receptors, including AGE-R1, AGE-R3, CD36, Scr-II, FEEL-1 and FEEL-2 (Sourris and Forbes, 2009). On the other hand, RAGE can interact with diverse ligands such as high mobility group box 1, some members of the S100 family, including S100A12, S100B and S100P, AGEs, amyloid and β-sheet fibrils (van Zoelen et al., 2009). Alternatively, it is yet to be determined whether the neutralizing antibody used in this study was a successful effector to fully neutralize the receptor and effectively block the interaction between AGEs and RAGE.

In response to stimuli, there are two major apoptotic pathways operating in vertebrates systems, namely extrinsic and intrinsic pathways. The extrinsic pathway is initiated by cell surface receptors like Fas and the intrinsic pathway involving mitochondria is induced by stimuli like DNA damage or UV irradiation (Wang and Youle, 2009). Activation of the mitochondrial pathway due to loss of the mitochondrial membrane potential results in escape of the pro-apoptotic molecules like cytochrome c and subsequently both second mitochondria-derived activator of caspase (Smac) and Omi that are capable of binding to inhibitors of apoptosis (IAPs) and initiate the activation of caspases (Wang and Youle, 2009). Activation of caspases, the initiator caspases (caspase-8 and -9) and the effector caspases (caspase-3, -6, and -7), is responsible for regulating the apoptosis program (Li and Yuan, 2008; Salvesen and Dixit, 1997). Following this thinking process, cytochrome c has been generally considered to be essential for caspase activation as suggested by earlier studies using a cell-free system by Xiaodong Wang's group (Liu et al., 1996). It is interesting, by different approaches, we showed that there was no detectable activation of caspase-3, caspase-5, caspase-8 and caspase-9 with the high concentration of AGEs treatment (Fig. 6D and Supplementary Fig. 4); however, the effects of AGEs-induced DNA nick and MOMP were successfully reduced by treatment with the pan-caspase inhibitor, Z-VAD-fmk. Our results indicated that certain caspase which is susceptible to inhibition by Z-VAD-fmk might be upstream of the events of DNA nick and MOMP in AGEs-mediated T cell apoptosis. Although it may not be exactly applied in the system of AGEs-mediated T cell apoptosis, there are indeed evidence suggesting that caspases may regulate or play upstream preceding the event of cytochrome c escape from the mitochondria (Lakhani et al., 2006; Li et al., 1998). Furthermore, a study from Arnoult et al. (Arnoult et al., 2003) demonstrated that the pan-caspase inhibitor Z-VAD-fmk effectively blocks AIF and endoG release from the mitochondrial both *in vitro* and *in vivo*.

In many tissue cells, glutathione depletion is one of the crucial events in apoptotic process induced by various apoptotic signals (Circu and Aw, 2008; Franco and Cidlowski, 2009). Our study showed that there was a significant increase of ROS in AGE-

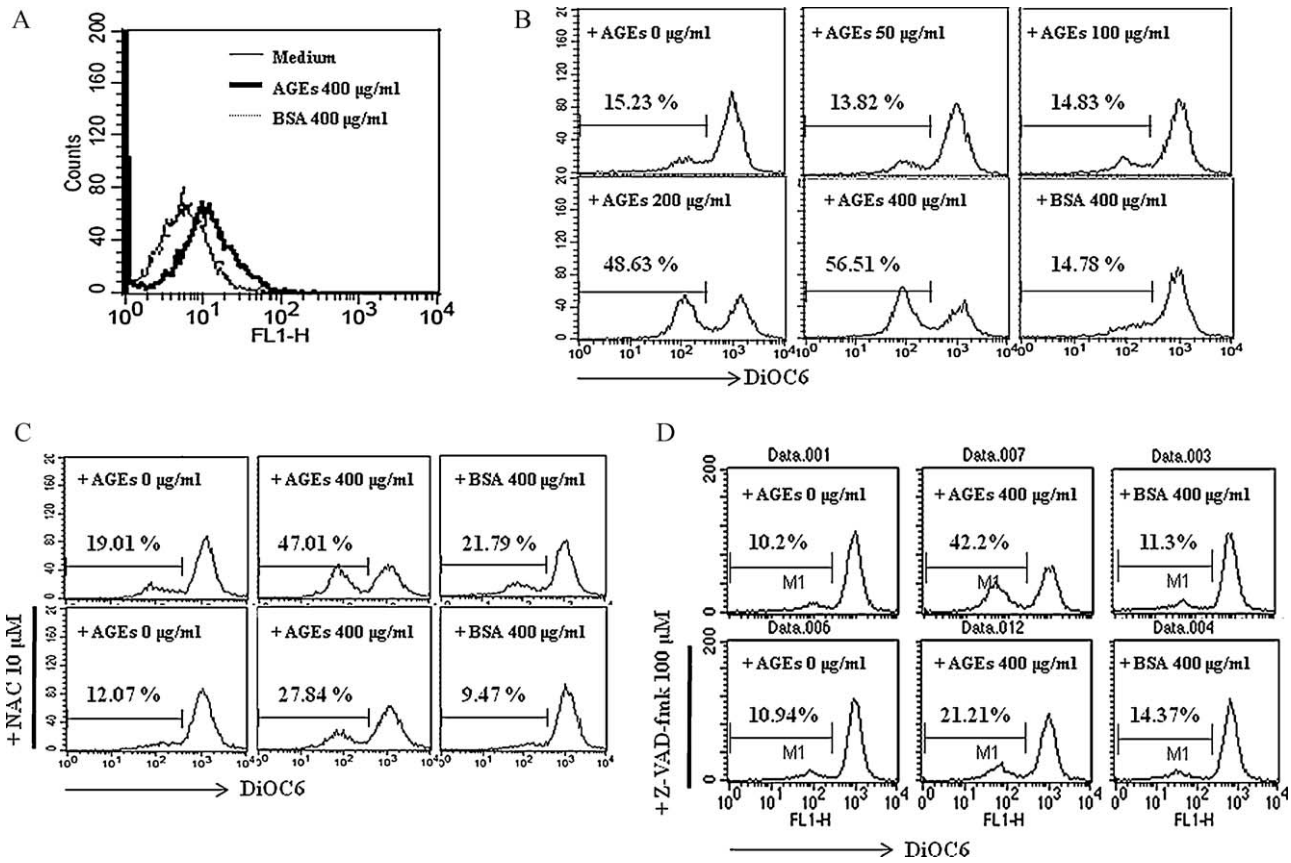

Fig. 7. AGEs induced ROS and activated MOMP that was susceptible to antioxidant and Z-VAD-fmk inhibition. (A) During the progression of AGEs-induced T cell apoptosis, the accumulation of ROS was evaluated by flow cytometry. Increased immunofluorescence intensity of ROS was detected in the AGEs-treated group. (B) The percentage of DiOC6-negative population increased under AGEs treatment. The results indicated that MOMP occurred during the progression of AGEs-induced T cell apoptosis. (C) Treatment with an antioxidant NAC inhibited AGEs-induced MOMP. (D) In the presence of the pan-caspase inhibitor Z-VAD-fmk, the AGEs-induced MOMP decreased. The results indicated that both oxidative stress and caspase-dependent mechanisms were required for the induction of MOMP by AGEs treatment in Jurkat T cells.

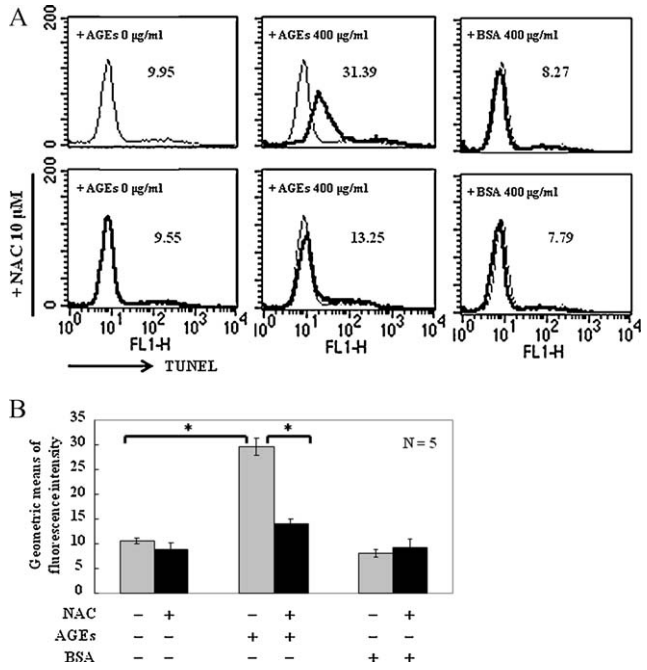

Fig. 8. The antioxidant NAC blocked AGEs-induced DNA nicking in Jurkat T cells. (A) The intensity of immunofluorescence increased after AGEs stimulation and the effect could be blocked by treatment with the antioxidant NAC. The results from five independent experiments were pooled together and analyzed (B). Values shown were geometric means of fluorescence intensity. $^{*}P < 0.05$.

induced T cell apoptosis. MOMP induced by AGEs could be blocked by supplementation of glutathion with an antioxidant NAC (Fig. 7C). In addition, treatment with NAC also prevented AGEs-induced DNA nicking. These results indicated the critical roles of oxidative stress in AGEs-induced T cell apoptosis. Surprisingly, the effects from glutathione depletion are not necessarily to be the same as those from ROS formation in apoptosis of lymphoid cells (Franco et al., 2007). Because the exact Z-VAD-fmk-susceptible caspase involved in AGEs-mediated T cell apoptosis is currently unclear, whether the oxidative stress also regulates the activation of this caspase-dependent pathway remains to be investigated. Altogether, the results presented in this report demonstrated that under the environment of high concentrations of AGEs, both immortalized and primary T cells might undergo apoptosis and the mechanisms involved were caspase-dependent and required depletion of glutathione and activation of the mitochondrial pathway. However, caspase-3, -5, -8 and -9 appeared not to participate in this apoptotic process.

A limitation of this report is that the experiments were conducted *in vitro* and may not really reflect *in vivo* situations. With regard to this concern, a recent report from Tsukamoto et al. (Tsukamoto et al., 2009) demonstrated that in a transgenic mice model, after thymectomy that prevents *de novo* production of T cells, the half-life of aged naïve T cells is about 3-fold longer than that of young cells and such an increased longevity appears to correlate with reduced expression of a pro-apoptotic molecule Bim. Nevertheless, the data provided in this report remain to be potentially useful and may lay the groundwork for future studies that more directly establish a link to ageing.

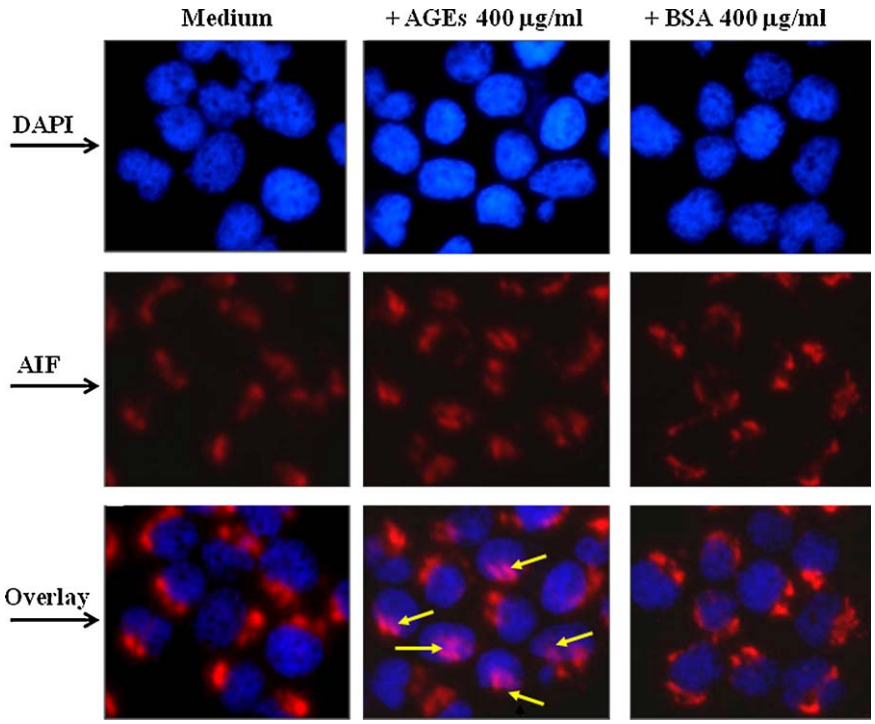

Fig. 9. Immunofluorescence analysis revealed that AIF migrated into the nucleus after AGEs stimulation. Jurkat T cells were treated with AGEs (400 μg/ml) for 5 days before immunofluorescence analysis with anti-AIF antibodies followed by detection with PE-conjugated secondary antibodies. Cells were counterstained with DAPI to visualize nuclei (blue staining). The localization of AIF was evaluated by a fluorescence microscope. There was a significant migration of AIF into the nucleus (pink staining pointed by arrows in the overlay pictures) after AGEs treatment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mad.2010.09.005.

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