J Korean Neurol Assoc / Volume 21 / August, 2003

PC12 세포에서 산화스트레스에 의한 베타-아밀로이드 펩티드의 세포독성

영남대학교 의과대학 생화학 · 분자생물학교실, 신경과학교실*

이재규 김찬석* 김태정 김병학 김성용 하정상* 김정희 김재룡

Involvements of Oxidative Stress in β-amyloid Peptide-induced Cytotoxicity in PC12 Cells

Jae-Kyu Lee, Chansok Kim*, Tae Jung Kim, Byung Hak Kim, Seongyong Kim, Jung Sang Hah*, Jung-Hye Kim, and Jae-Ryong Kim

Department of Biochemistry and Molecular Biology, Department of Neurology College of Medicine, Yeungnam University*

Background: Alzheimer's disease is a neurodegenerative disorder characterized by the extracellular deposition of β-amyloid peptide(Aβ) in the brain, presumed to play a pathogenic role. However, the precise molecular mechanisms of its neurotoxicity are not fully understood. **Methods**: Aβ-mediated cytotoxicity in neuronal cell lines (PC12, SH-SY5Y, IMR32, and U87) was measured by an MTT assay. NF-κB activation by Aβ was examined by a luciferase assay and apoptosis induced by Aβ was measured by cytoplasmic DNA fragmentations. **Results**: Aβ cytotoxicity in the tested cell lines was more prominent in the absence of serum than in the presence of serum in culture media. PC12 cells showed the highest sensitivity to Aβ cytotoxicity among the cell lines. The Aβ(25-35) cytotoxicity in PC12 cells was increased in a dose-dependent manner. For convincing oxidative stress involved in Aβ cytotoxicity, antioxidants such as DTT, GSH, vitamin C, or NAC were pretreated. GSH protected PC12 cells from Aβ cytotoxicity, but DTT or NAC did not. Aβ (25-35) treatment to PC12 cells increased the NF-κB activity in a dose-dependent manner. Cytoplasmic DNA fragmentations, one of the apoptotic indicators, were increased at lower concentrations of Aβ(25-35) from 0.01 to 0.1 μM, however, dose-dependent increments of DNA fragmentations were not observed at higher concentrations from 1 to 10 μM. **Conclusions**: From these results, Aβ-induced cytotoxicity in PC12 cells might be mediated by oxidative stress.
J Korean Neurol Assoc 21(4):401~407, 2003

Key Words: Alzheimer disease, Amyloid beta-protein, Oxidative stress, PC12 cells

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that results in dysfunction of learning and memory and the most common senile dementia in the elderly. Pathologically, AD patients show loss of neurons in temporal lobes, insoluble aggregates (senile plaques or amyloid plaques) consisting of β-amyloid peptides (Aβ) and cytoskeletal proteins, and neurofilament tangles.[1] Loss of neurons in AD might result from oxidative stress due to changes in cellular redox status,[2-4] abnormalities in intracellular calcium homeostasis by Aβ,[5, 6] and/or Tau in amyloid plaques.[7] Substantial evidence indicates that the oxidative stress involves mainly in pathogenesis of AD; increases in iron, aluminum, or mercury producing free radicals in AD brains,[8, 9] oxidations of lipids,[10-12] proteins[13, 14] and DNA,[15] and increments of advanced glycation end product,[16] peroxynitrite, heme oxygenase-1, or superoxide dismutase-1 in neurofilament tangles or senile plaques.[17] Aβ, one of the components in the senile plaques, was known to produce hydrogen peroxide in cell free systems.[18, 19]

Manuscript received July 19, 2003
Accepted in final form March 20, 2003
* Address for correspondence
Jae-Ryong Kim, M.D.
Department of Biochemistry and Molecular Biology, College of Medicine, Yeungnam University
317-1 Daemyong-dong, Daegu, 705-717, Korea
Tel : +82-53-620-4342 Fax : +82-53-654-6651
E-mail : kimjr@med.yu.ac.kr

Aβ is a component of amyloid plaques and a hydrophobic peptide with 40-43 amino acid residues. [20] Aβis produced from amyloid precursor protein (APP) encoded in chromosome[21] by several cleavage enzymes such as BACE.[21,22] Genetic, neuropathological, and biochemical evidence indicates that Aβplays an important role in the early pathogenesis in AD.[23] Cytotoxicity induced by Aβis observed in several neuronal cell lines such as rat pheochromocytoma cell line (PC12), clonal CNS cell line (B12), and primary neuronal cells.[18,19]

Oxidative stress results when oxidant production overrides the antioxidant capacity of the cell to prevent oxidative injury.[24] Cellular oxidative stress can modify activities of various signaling molecules, such as NF-κB, a redox-regulated transcription factor, and result in alterations in cellular growth and death.[25] Aβ-induced NF-κB activations were reported in B12 cells,[18] and rat primary cerebellar neuronal cell and inhibited by treatment of antioxidants such as PDTC and catalase.[26]

Cell death by Aβis controversial to undergo necrosis [27] or apoptosis.[28] Recently, many evidence, such as increased DNA fragmentations,[28] activation of caspases, [29,30] inhibition of Aβcytotoxicity by caspase inhibitors,[31] and induction of genes involved in apoptosis by Aβ[32] showed the relations of apoptosis to Aβcytotoxicity.

In order to clarify that Aβ-mediated cytotoxicity results from oxidative stress due to impairments of cellular redox status, we have measured the Aβcytotoxicity in several neuronal cell lines and effects of antioxidants on Aβcytotoxicity. And NF-κB activation and cytoplasmic DNA fragmentations by Aβwere also investigated.

Materials and Methods

1. Materials

The Aβ(25-35) peptide, its reverse peptide rAβ(35-25) and the Aβ(1-40) peptide were purchased from Bachem AG (Switzland) or American Peptide Company Inc. (USA). The peptides were dissolved in distilled water or 0.1 % trifluoroacetic acid, and stored at -70 ℃ prior to experiments. Dulbecco's modified Eagles medium (DMEM), modified Eagles medium (MEM), penicillin/streptomycin/amphotericin B antibiotics solution, fetal bovine serum (FBS), horse serum (HS), and Lipofectamine 2000 were from Life Technologies Inc. (USA). Bovine calf serum (BCS) was from Hyclone Laboratories Inc. (USA). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), reduced glutathione (GSH), ascorbate, dithiothreitol (DTT), N-acetylcysteine (NAC), and dimethylsulfoxide (DMSO) were from Sigma-Aldrich Co. (USA). A luciferase assay kit was obtained from Promega Corp. (USA).

2. Cell culture and maintenance

Rat pheochromocytoma cell line, PC12, was cultured in DMEM media containing 5 % BCS, 10 % HS, and antibiotics at 37 ℃ in an atmosphere of humidified 95 % air and 5 % CO_2. Human neuronal cell line, IMR32, was cultured in MEM media containing 10% FBS and antibiotics. Human neuronal cell line, SH-SY5Y and human glial cell line U87 were maintained in DMEM containing 10 % FBS and antibiotics.

3. MTT assay

PC12, SH-SY5Y, IMR32, and U87 cells were seeded in 96 well plates (20,000/well) and incubated for 24 hours at 37 ℃ in an atmosphere of humidified 95 % air and 5 % CO_2. After aspirating the media, 100 μl of media with or without 10 % FBS was added into the cells. Following incubation for 24 hours, the media was removed and the cells were treated with the indicated concentrations of peptides in 50 μl of MEM or MEM+ 10 % FBS for 48 hours. Cell viability was measured by MTT assay. Briefly, 25 μl of MTT (50 mM in PBS) was added into the cells and incubated for 3 hours. The media was aspirated carefully and the crystals of MTT-formazan were dissolved with 100 μl of DMSO. Cell viability was estimated by measuring the absorbance at 570 nm with a microplate reader (BioRad Laboratories, USA).

4. Effects of antioxidants on Aβ-mediated cytotoxicity

PC12 cells were pretreated with 2 mM of DTT, GSH, ascorbate, or NAC in 40 μl of MEM for 1 hour and then final 10 μM Aβ(25-35) or rAβ(35-25) was added. After incubation for 48 hours, cell survivals were measured by MTT assay.

5. Measurement of NF-κB activation

PC12 cells were seeded in 60 mm culture dishes (3×10^6/dish) and incubated for 24 hours. Plasmid DNAs of

luciferase containing NF-κB binding elements (NF-κB-Luc) were transfected into cells using Lipofectamine 2000 in accordance with manufacturer's protocols. After 24 hours, cells were harvested, dispensed into 24 well plates, and further incubated for 24 hours. The cells were treated with the indicated concentrations of Aβ(25-35) or rAβ(35-25) for 24 hours. After harvesting, cell extracts were prepared with 50 μl of lysis buffer (25 mM Tris-HCl, pH 7.4, 1 % Triton X-100, 150 mM NaCl, 100 μM AEBSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin). The activities of luciferase were measured with a luciferase assay kit and protein concentrations were determined with a BCA protein assay kit (Pierce, USA) using bovine serum albumin as a standard.

6. Cytoplasmic DNA fragmentations

Cytoplasmic DNAs were prepared for the evaluation of DNA fragmentations. PC12 cells treated with the indicated concentrations of Aβ(25-35) or rAβ(35-25) for 24 hours were harvested and incubated with 200 μl DNA isolation buffer (5 mM Tris-HCl, pH 7.4, 20 mM EDTA, 0.5 % Triton X-100, 1 mM PMSF) for 30 min on ice with an intermittent vortexing. Following centrifugation of the cell lysates at 15,000 rpm for 5 min, the supernatants were transferred to new tubes and measured protein concentration with a BCA assay kit. The supernatants with the same amount of protein were extracted with tris-saturated phenol, phenol/chloroform, and chloroform and DNAs were precipitated. The DNAs were dissolved in 10 μl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing RNAse (1 mg/ml), separated on a 2 % agarose gel, and analyzed by staining with ethidium bromide.

7. Statistical analysis

Values were expressed by mean±SD. Student's *t* test

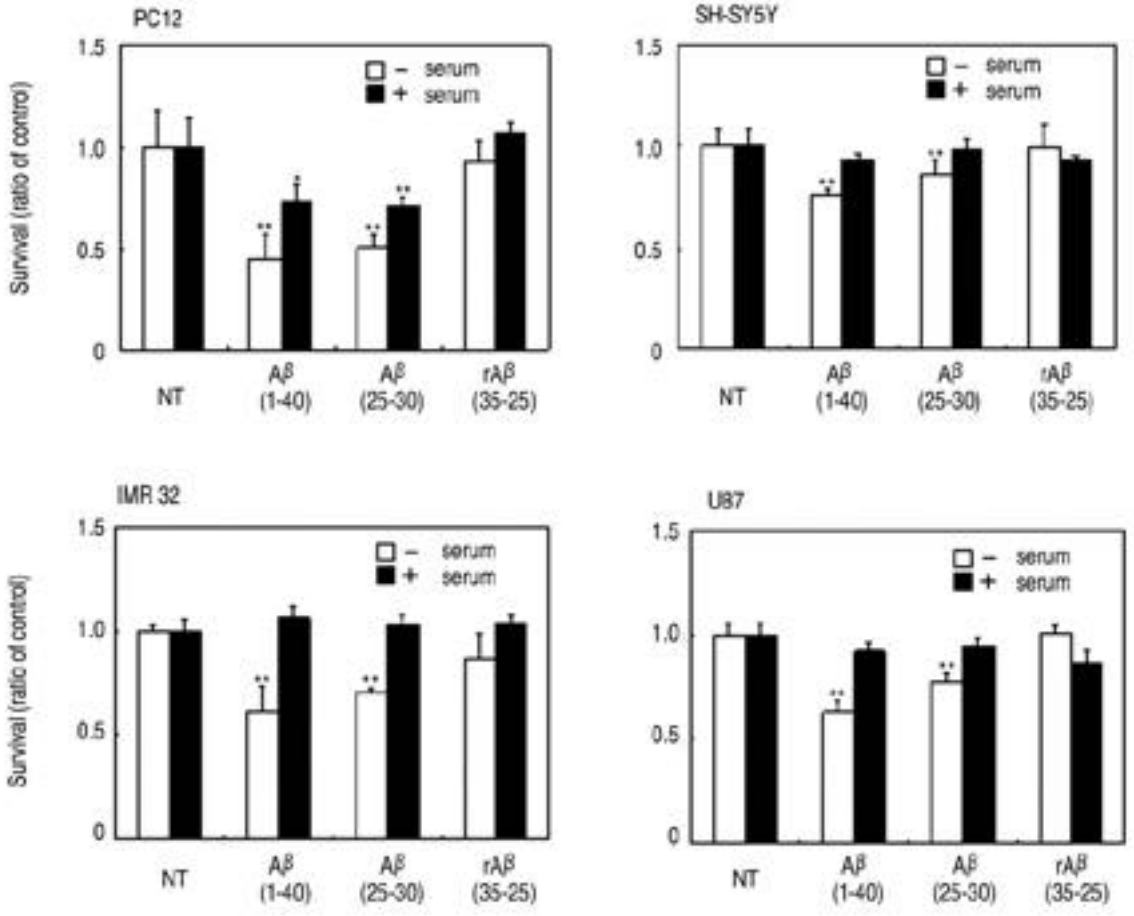

Figure 1. Aβcytotoxicity in PC12, SH-SY5Y, IMR32, and U87. Cells are treated with 10 μM of Aβpeptides in the presence (■) or absence (□) of serum for 48 hours and cell survivals are measured by MTT assay. Values are mean±SD from triplicate wells of 3 independent experiments. Statistical significance is estimated by Student's *t* test between the Aβtreated and the Aβuntreated (NT) cells (**, P<0.01; *, P<0.05).

was used to compare the mean differences between the groups. P-value of less than 0.05 was considered as statistically significant.

Results

A β cytotoxicity was measured in PC12, SH-SY5Y, IMR32, and U87 cells treated with 10 μM of A β(25-35), rA β(35-25), or A β(1-40) (Fig. 1). Cell survivals were decreased by the treatments of A β(25-35) and A β(1-40) in the tested cells significantly (P<0.01). A β cytotoxicity was more prominent in the absence of FBS than in the presence of FBS. rA β(35-25) had no effects on cell death. PC12 cells showed the highest sensitivity to A β cytotoxicity among the cells. Cell survival of PC12 treated with A β was decreased in a dose dependent manner (Fig. 2). It has been reported that the A β-mediated cytotoxicity was observed only by the aggregated form of peptides, which was prepared by dissolving the peptides in distilled water and incubating for 1 to 5 days at 37 ℃. [33, 34] The A β(25-35) peptide in distilled water was

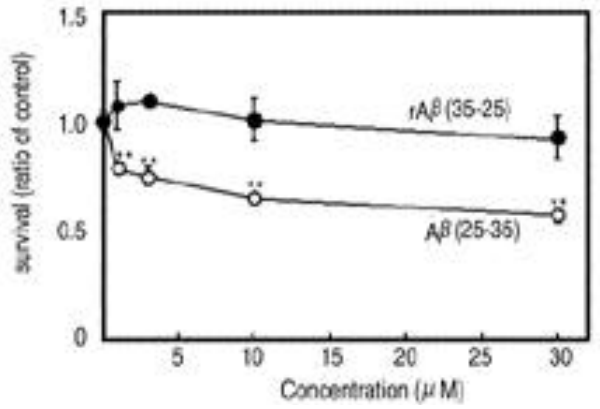

Figure 2. Dose-dependent increases in A β cytotoxicity in PC12 cells. Cells are treated with the indicated concentrations of A β (25-35) (○) or rA β(35-25) (●) in the absence of serum for 48 hours and cell survivals are measured by MTT assay. Values are mean±SD of triplicate samples of 3 independent experiments. Statistical significance is estimated by Student's *t* test between the A β(25-35) and the rA β(35-25) -treated cells (**, P<0.01; *, P<0.05).

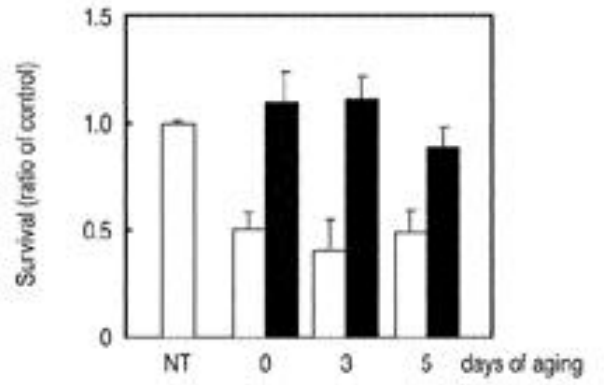

Figure 3. Effects of aging of A β peptides on cytotoxicity in PC12. The peptides are aged by incubation for 3 or 5 days at 37 ℃. Cells are treated with 10 μM of A β(25-35) (□) or rA β(35-25) (■) for 48 hours in the absence of serum and cell survivals are measured by MTT assay. Values are mean± SD of triplicates in 2 independent experiments. NT, not treated with peptides.

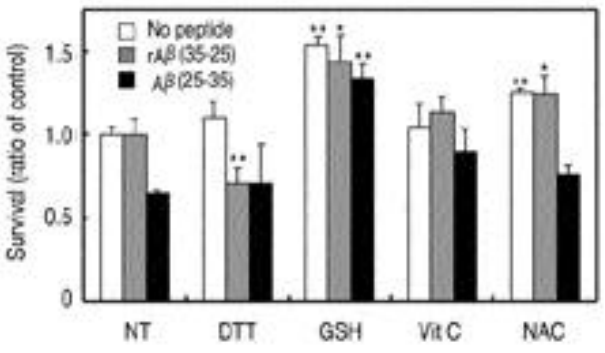

Figure 4. Effects of antioxidants on A β cytotoxicity in PC12. Cells are pretreated with antioxidants at the concentration of 2 mM for 1 h and further treated with 10 μM peptides in the absence of serum for 48 hours. Cell survivals are measured by MTT assay. Values are mean±SD of triplicates of 3 independent experiments. Statistical significance is estimated by Student's *t* test between the antioxidant-pretreated and the antioxidant-untreated cells (**, P<0.01; *, P<0.05). NT, not treated with antioxidants, DTT, dithiothreitol, GSH, glutathione, Vit C, vitamin C, NAC, N-acetylcysteine.

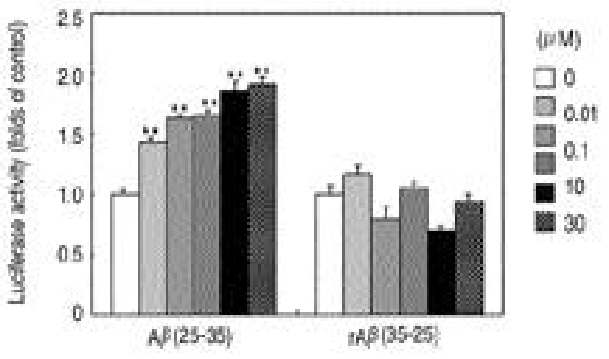

Figure 5. NF-κB activation by A β(25-35) in PC12 cells. Cells are transfected with a NF-kB-Luc plasmid using Lipofectamine 2000 and incubated with the increasing concentrations of the peptides in the absence of serum. Following 24 hours, cells are harvested and proteins are extracted. Luciferase activities and protein concentrations are measured. NF-κB activation is expressed by folds of control after normalizing with protein concentration. Values are mean±SD of triplicates of 2 independent experiments. Statistical significance is estimated by Student's *t* test between the A β untreated and the A β treated cells (**, P<0.01; *, P<0.05).

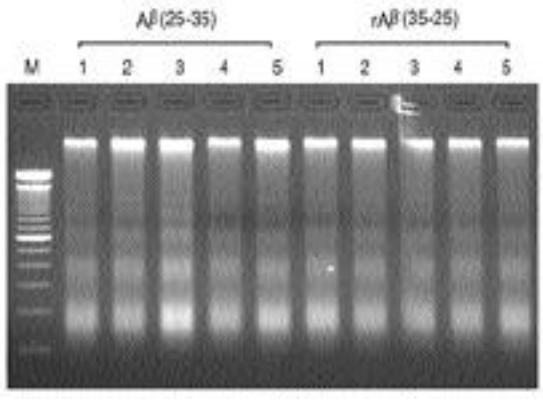

Figure 6. Cytoplasmic DNA fragmentations in PC12 cells treated with A β peptides. PC12 cells are treated with increasing concentrations of the peptides and cytoplasmic DNAs are isolated. DNAs are separated on a 2 % agarose gel and stained with ethidium bromide. This shows representative data from 3 independent experiments. M, 100 bp ladder; 1, 0 μM; 2, 0.01 μM; 3, 0.1 μM; 4, 1 μM; 5, 10 μM of the peptides.

incubated for 0, 3 or 5 days at 37 ℃, and then treated to PC12 cells. There were no differences in cytotoxic effects of the A β(25-35) peptides aged for 0, 3, or 5 days (Fig. 3). The rA β(35-25) peptide showed no cytotoxicity whether it was aged or not.

Next, we examined effects of antioxidants on the A β cytotoxicity. PC12 cells were incubated with 2 mM of DTT, GSH, vitamin C, or NAC prior to treatment of 10 μM A β(25-35). Pretreatment of GSH protected the cells from A β cytotoxicity significantly ($P<0.01$), but DTT or NAC did not (Fig. 4).

To confirm that the A β(25-35)-mediated cytotoxicity is associated with activation of NF-κB, a plasmid containing NF-κB binding element and luciferase was transfected into PC12 cells and NF-κB activation by A β was analyzed by measuring the luciferase activity. PC12 cells treated with A β showed the increments of NF-κB activation in a dose dependent manner (Fig. 5).

In order to identify whether A β cytotoxicity might undergo apoptosis or necrosis, DNA fragmentations were analyzed. Although DNA fragmentations were increased by the treatment of 0.01 and 0.1 μM of A β(25-35), dose-dependent increments of DNA fragmentations could not be observed at 1 and 10 μM of A β(25-35) (Fig. 6).

Discussion

A β cytotoxicity in PC12, SH-SY5Y, IMR32, and U87 showed clear differences in the presence or in the absence of serum. Behl et al.[18,35] reported that in order to observe A β cytotoxicity in neuronal cells, fetal bovine serum should be dialyzed and added to the concentration of 5 % in the media. However, some reports [33,36] showed that the A β cytotoxicity was observed in the presence of serum, which might suggest that serum does not affect the A β cytotoxicity. In our conditions, the absence of serum increases A β cytotoxicity in all tested cell lines compared to the presence of serum. Therefore, some components in serum might involve in the protection of A β cytotoxicity. The dose-dependent increments of A β cytotoxicity in PC12 cells suggest that the A β toxicity is specific to the A β(25-35) peptide. The concentrations of A β peptides to induce cytotoxicity in neuronal cells varied from nanomolar[35] to micromolar depending on the cell types. Interestingly, many reports[35-38] showed that the cell survival maintained at the level of 50% even though A β was treated up to 100 μM. Our results, 56% survival at 30 μM A β(25-35) and plateau effects of toxicity from 3 to 30 μM, also coincide with other reports. It might be possible to increase the cytotoxicity by the treatment of much higher concentration of A β However, further study will be needed for this finding.

Aggregation of A β peptides has been reported to be critical for the A β-mediated cytotoxicity.[33,34] Our results showed no differences in A β cytotoxicity depending on aging of the peptides, which might be different to other reports. However, we could observe that the A β(25-35) peptide was already aggregated prior to aging and rA β(35-25) did not form aggregates by SDS-polyacrylamide gel electrophoresis (data not shown).

Many reports indicated that A β cytotoxicity might result from oxidative stress [2,3,4] and antioxidants such as catalase,[18] vitamin E,[35,39] 2-mercaptoethanol,[40] and melatonin,[41] protected cells from A β-mediated cytotoxicity. However, other antioxidants, such as propyl gallate, trolox, probucol, and promethazine did show no protective effects on A β cytotoxicity.[37] The fact that protective effects on A β-mediated cytotoxicity were quite different depending on types of antioxidants was also observed in our experiments. GSH had protective effects on A β cytotoxicity in PC12 cells. However, DTT and NAC showed no protective effects. GSH, DTT, NAC, and β-mercaptoethanol were expected to protect A β cytotoxicity because all of these antioxidants contain sulfhydryl groups to reduce reactive oxygen species. However, the fact that protective effect of these antioxidants against A β cytotoxicity was quite different would need further experiments.

In conclusion, A β-mediated cytotoxicity in PC12 cells might result from oxidative stress, which might be associated with NF-κB activation and apoptosis at low concentrations of A β

Acknowledgement

This work was supported by grant No. R01-1999-000-00087 from the Korea Science & Engineering Foundation.

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