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Aspartame-induced apoptosis in PC12 cells

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Abstract Aspartame is an artificial sweetener added to many low-calorie foods. The safety of aspartame remains controversial even though there are many studies on its risks. In this study, to understand the physiological effects of trace amounts of artificial sweeteners on cells, the effects of aspartame on apoptosis were investigated using a PC12 cell system. In addition, the mechanism of apoptosis induced by aspartame in PC12 cells and effects on apoptotic factors such as cytochrome c, apoptosis-inducing factor, and caspase family proteins were studied by western blotting and RT-PCR.

Aspartame-induced apoptosis in PC12 cells in a dose-dependent manner. In addition, aspartame exposure increased the expressions of caspases 8 and 9 and cytochrome c. These results indicate that aspartame induces apoptosis mainly via mitochondrial pathway involved in apoptosis due to oxygen toxicity.

Keyword: Aspartame; Apoptosis; Caspases; AIF; PC12; Cytochrome c

1. Introduction

Chemical engineering has led to the development of artificial sweeteners, which are used as alternatives to sugar. Aspartame consists of 2 amino acids in addition to methanol, i.e., aspartic acid and phenylalanine. The American Food and Drug Administration and the European Food Safety Authority established acceptable daily intake levels of 50 and 40 mg aspartame/(kg bw•day), respectively. The LD50 of aspartame in mice and rats is >5 g/kg (Kotsonis and Hjelle, 1996). Aspartame is used in low-calorie soft drinks, foods, and sweeteners because aspartame is approximately 200 times sweeter than sugar. Aspartame is a low calorie option for people who should or need to limit their sugar intake. Furthermore, unlike sugar, aspartame does not contribute to tooth decay. Aspartame is currently used in over 6,000 products in over 120 countries worldwide. As aspartame is an artificial sweetener used in food products, many safety tests have been carried out. Ishii (1981) reported that aspartame does not cause brain cancer in rats. Butchko et al. (2002) reviewed studies regarding the safety of aspartame and concluded that it is safe. However, Olney et al. (1996) reported that aspartame might be associated with an increased incidence of brain tumors. Gurney et al. (1997) have reported that aspartame consumption is related to brain tumor risk in children. In addition, aspartame is reported to cause leukemia (Soffritti et al., 2005; 2006; 2007) as well as liver and lung cancers (Soffritti et al., 2010). **Furthermore risk of lymphoma and leukemia in peoples who drinking light beverages added with artificial sweetener such as aspartame had been reported (Schernhammer et al., 2012).** Therefore, the safety of aspartame remains controversial.

Thus far, animal experiments have been carried out to assess the risks of food additives on biological systems. **Long term bioassays using experimental animals have**

been the gold standard of the last 40 years in order to assess the toxicity/carcinogenicity of chemical agents. However, unfortunately these experiments require large amounts of resources and time to understand the effects of an additive. The risks associated with trace amounts of chemicals can also be predicted from dose-response experiments using *in vitro* system because the toxicity is difficult to be investigated using trace amounts in animal experiments.

The present study aimed to clarify whether trace amounts of aspartame affects organisms; a cultured cell system was used. PC12 cells, rat adrenal pheochromocytoma cells, induce apoptosis by removing the serum from the whole culture medium; moreover, apoptotic PC12 cells exhibit DNA fragmentation (Batistatou and Greene, 1991; 1993). When electrophoresis is performed using DNA from cell-induced apoptosis, a ladder pattern due to DNA fragments is observed. Thus, PC12 cells are a very useful model for studying the mechanisms of the induction and inhibition of apoptosis (Maroto and Perez-Polo, 1997; Gollapudi and Oblinger, 1999).

Apoptosis is a physiological cell-death mechanism commonly associated with programmed events, including morphological and biochemical changes, which are necessary for the differentiation and development of organs and organisms (Maroto and Perez-Polo, 1997). There are 2 major apoptotic pathways in cells: the death receptor-mediated pathway (Schmitz et al., 2000; DeBiasi et al., 2010) and the mitochondria-mediated pathway (Cheng et al., 2008). Moreover, the mitochondrial pathway can be divided into caspase-dependent and caspase-independent pathways. The mitochondrial caspase-dependent pathway is regulated by members of the Bcl-2 family, which have either anti-apoptotic (e.g., Bcl-2) or pro-apoptotic (e.g., Bax and Bad) properties (Tsujimoto and Shimizu, 2000; Adams and Cory, 2001). The pro-apoptotic

proteins of the Bcl-2 family execute their function in the mitochondria (Adams and Cory, 2001). After apoptotic stimuli, the pro-apoptotic proteins trigger the release of cytochrome c from mitochondria, which accelerates the activation of caspase 9, ultimately leading to cell death (Emerit et al., 2004). On the other hand, apoptosis-inducing factor (AIF) is released from mitochondria when cells experience serious oxidative stress (Natarajan and Becker, 2012). AIF was the first mitochondrial protein shown to mediate cell death without the involvement of caspases (Susin et al., 1996; 1999). AIF translocates to the nucleus and controls nuclear functions such as chromatin condensation and large-scale DNA degradation (Susin et al., 1996).

We developed a novel risk evaluation method for trace chemical substances in organisms in our laboratory using a PC12 cell system (Yamanoshita et al., 2000). Kawakami et al. (2008) demonstrated the mechanism of apoptosis induced by copper in PC12 cells. Nonylphenol, an endocrine disrupter, enhances apoptosis (Aoki et al., 2004). However, little is known about the effects of aspartame on apoptosis. Therefore, we aimed to establish a system for evaluating trace amounts of aspartame, a food additive using the PC12 cell system. First, the cell toxicity of PC12 cells exposed to aspartame is measured by trypan blue exclusion and lactate dehydrogenase (LDH) assays. Then, to confirm whether aspartame influences apoptosis, DNA fragmentation analyses in PC12 cells were carried out. Thus, in this study changes in apoptotic factors such as cytochrome c, AIF, and caspases in PC12 cells exposed to aspartame were measured by western blotting and RT-PCR to clarify the apoptotic pathway induced by aspartame.

2. Materials and Methods

2.1. *Materials*

PC12 cells were purchased from the American Type Culture Collection (USA and Canada). Dulbecco's modified Eagle's medium (DMEM), ribonuclease A, streptavidin-conjugated peroxidase, peroxidase-conjugated avidin, ethidium bromide and o-phenylenediamine dihydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). Aspartame was obtained from Tokyo Chemical Industry (Tokyo, Japan). Terminal deoxynucleotidyl transferase (TdT) was obtained from Toyobo (Osaka, Japan). The monoclonal antibody against cytochrome c and cytochrome c Release Apoptosis Assay kit were from Calbiochem (Darmstadt, Germany). The polyclonal antibody against AIF was from BD Biosciences Pharmingen (San Jose, CA, USA). The biotinylated donkey anti-rabbit immunoglobulin, biotinylated goat anti-mouse immunoglobulin, and ECL western blotting detection reagent were from Amersham Pharmacia Biotech (Buckinghamshire, England). Fetal bovine serum (FBS) was purchased from HyClone (Rockville, MD, USA). The SV total RNA isolation system, non-radioactive cytotoxicity assay kit, RT-PCR kit, 6× loading dye, and DNA markers were from Promega (Madison, WI, USA). The high pure PCR template preparation kit, biotin-16-2'-deoxy-uridine-5'-triphosphate, proteinase K, and blocking reagent were from Roche Diagnostics (Mannheim, Germany). Trypan blue stain solution (0.5%) was purchased from Nacalai Tesque (Kyoto, Japan). Other chemicals were of analytical reagent grade.

2.2. Cell culture

PC12 cells were maintained in DMEM supplemented with 10% FBS in a humidified incubator at 37°C with 5% CO₂. The cells were preincubated in 25 cm² flasks overnight,

and the medium was then replaced with DMEM without or with various concentrations of aspartame. Aspartame was resolved in water to prepare as each concentration.

2.3. *Cell viability*

Cell viability was measured using the trypan blue exclusion assay. PC12 cells were incubated in the medium with 0–8 µg/mL aspartame for 72 h. After cultivation, the cells were stained with 0.25% trypan blue solution in phosphate-buffered saline (PBS). The total number of cells and trypan blue-stained cells were counted. Cell viability was expressed as the percentage relative to the total cell number. Each experiment was repeated at least 3 times, and triplicate samples were counted for each condition.

2.4. *Cytotoxicity assay*

The cytotoxicity of aspartame was estimated using a non-radioactive cytotoxicity assay kit on the basis of the activity of LDH, which was released from the dead cells into the medium. Aliquots of the cultured PC12 media containing 0–8 µg/mL aspartame were collected and transferred to multi-titer plates. Substrate mixture containing tetrazolium salts was added, and the cells were incubated for 0.5 h. The absorbance of formazan dye was measured at 495 nm by a Micro Plate Reader model 450 (Bio-Rad, USA). The cytotoxicity of aspartame was expressed as the activity relative to that of the control medium.

2.5. *Isolation of genomic DNA from PC12 cells*

PC12 cells were cultured in the medium containing 0–8 µg/mL aspartame for 72 h. After incubation with aspartame, the cells were harvested using a scraper. The harvested cells were washed with 3 mL PBS. Genomic DNA was isolated using a high pure PCR template preparation kit according to the manufacturer's instructions. Finally, 500 µL ethanol and 20 µL 3 M NaOAc buffer (pH 4.5) were added for ethanol precipitation, and the solution was allowed to stand overnight at room temperature.

2.6. Genomic DNA electrophoresis

The ladder pattern of DNA was analyzed by agarose gel electrophoresis. To adjust the DNA concentration, the absorbance of genomic DNA from PC12 cells at 280 nm resolved in 80 µL Tris-borate buffer (pH 9.0) including 1 mM EDTA (1× TBE), was measured with a Personal Spectrum Monitor (Gene Quant Pro, GE, USA). To adjust all samples to the same DNA concentration, 1× TBE was added to all samples. Approximately 5 µg DNA with loading dye was subjected for electrophoresis on 1.5% agarose gel. Electrophoresis was carried out for 40 min at 100 V with a submarine-type electrophoresis system (Mupid-ex, ADVANCE, Tokyo, Japan). The electrophoresed gel was then soaked in ethidium bromide solution for 15 min in a darkroom. A photograph of the agarose gel was subsequently taken to determine the cell death situation under UV illumination with a ChemiDoc XRS (Bio-Rad, USA).

2.7. Quantification of DNA fragmentation in PC12 cells using the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method

After cultivating the PC12 cells in media containing 0–8 µg/mL aspartame for 72 h, genomic DNA was isolated as described above. The obtained DNA was resuspended in

1× TBE buffer, and equal amounts of DNA were placed into 96-well plates. The DNA fragmentation in PC12 cells was quantified using the method of Kurasaki et al. (2012).

2.8. RT-PCR

The expressions of caspase 8 and 9 mRNA were detected using the access RT-PCR kit and access RT-PCR introductory kit according to the manufacturers' instructions. The total RNA of PC12 cells cultured in the serum/serum-free media containing 0–8 µg/mL aspartame for 72 h was prepared using the SV Total RNA Isolation kit. The PCR primers of caspase 8 and 9 were synthesized according to the DNA sequences of caspase 8 and 9 described by Kawakami et al. (2008).

The PCR conditions were as follows: 48°C for 45 min and 94°C for 2 min; The cycles were carried out at 94°C for 30s, 60°C for 1 min, and 68°C for 2 min. Final extension was carried out for 7 min at 68°C. The numbers of cycles performed were 30 and 40. An annealing temperature of 60°C was used for caspases 8 and 9 (Kawakami et al., 2008).

PCR for β-actin was chosen as an internal control. The PCR products were verified using a DNA 7500 assay with an Agilent 2100 Bio Analyzer (Wilmington, DE USA) according to the instruction manual (Kawakami et al., 2008).

2.9. Western blot analysis using anti-cytochrome c and anti-AIF antibodies

Leaked cytochrome c and AIF from cytosol were isolated using the cytochrome c Release Apoptosis Assay kit (Calbiochem, Germany). PC12 cells were cultured in 5 mL DMEM with 10% FBS containing 0–8 µg/mL aspartame for 72 h. The cells were

subsequently harvested using a scraper. The obtained cells were then centrifuged at 1,800 rpm for 5 min to remove the supernatant. Then, 10 mL ice-cold PBS was added, and the mixture was centrifuged again at 1,800 rpm for 5 min. After removing the supernatants, the cells were resuspended in 500 μ L Cytosol Extraction Buffer Mix (1 \times Cytosol Extraction Buffer, 500 \times Protease Inhibitor Cocktail and 1 M dithiothreitol). The mixture was allowed to stand on ice for 10 min, and the cells were then disrupted by specified glassware. To remove the unbroken cells, the lysed cells were centrifuged at 3,000 rpm for 5 min. The supernatant was transferred into a 1.5-mL tube and centrifuged at 11,000 rpm for 30 min. The final supernatant was collected as the cytochrome c fraction.

The prepared fractions mentioned above were separated by polyacrylamide gel electrophoresis (Laemmli, 1970). The electrophoresed proteins were transferred to nitrocellulose membranes using a semidry blotting system (Type-AE6678; ATTO, Japan). The protein band responded to using antibody was detected according to the methods of Sun et al. (2012). In briefly, the membranes were incubated overnight at 4°C in blocking reagent. The membranes were then incubated for 90 min at room temperature with primary antibodies (dilution, 1:200). The membranes were washed 3 times and incubated for 1 h at room temperature with secondary antibody (dilution, 1:200). Finally, streptavidin-conjugated peroxidase (diluted 1:400 with the same blocking buffer) was added, and the membrane was incubated for 1 h at room temperature. Protein bands were detected with an enhanced chemiluminescence system. The image of the detected bands was analyzed using ChemiDoc XRS (Bio-Rad, USA).

2.10. Statistical analysis

All values are expressed as mean \pm SEM. Statistical analyses were performed by one-way analysis of variance, followed by Fisher's test.

3. Results

3.1. Cell viability and LDH activity

To examine whether aspartame affects cells, cell viability was measured by trypan blue staining after PC12 cells were exposed to 0–8 $\mu\text{g/mL}$ aspartame for 72 h.

As shown in Fig. 1A, the viability of the cells cultured in media containing aspartame decreased in a concentration-dependent manner. Cell viability decreased significantly after exposure to $>1 \mu\text{g/mL}$ aspartame.

In addition, to reconfirm the cytotoxicity caused by aspartame using trypan blue staining, the LDH activity in the cultured media was measured; dead cells released LDH into the medium (Fig. 1B).

The cytotoxicity of aspartame was corroborated by the results regarding LDH activity. As shown in Fig. 1B, the LDH released from the cells exposed to $>0.001 \mu\text{g/mL}$ aspartame was significantly greater than that of the control cells. These results indicate that aspartame exhibits cell toxicity at low concentrations.

3.1. Detection of DNA fragmentation by agarose gel electrophoresis

To investigate whether aspartame induces apoptosis, the DNA fragmentation of PC12 cells cultured in media containing 0–8 $\mu\text{g/mL}$ aspartame was observed (Fig. 2). The morphological characteristics of apoptosis are frequently accompanied by the cleavage

of DNA into 180–200-bp fragments. These oligonucleosomal-sized fragments can be visualized as a characteristic DNA ladder following agarose gel electrophoresis (Woodgate et al., 1999).

The DNA ladder pattern was observed in the cells cultured in the serum media containing >1 ng/mL aspartame (Fig. 2). Furthermore, on the basis of the photograph the DNA ladder increased with increasing aspartame in a concentration-dependent manner. These results indicate that aspartame itself induced apoptosis.

3.2. *Quantification of DNA damage using the TUNEL method*

DNA is fragmented by the action of endonucleases. The TUNEL method specifically labels the DNA ends generated by endonucleases activity (Gollapudi and Oblinger, 1999). The DNA ladder can be quantified using the TUNEL method to evaluate the degree of apoptosis in cells.

As shown in Fig. 3, the TUNEL signals increased significantly in the cells cultured in the media containing >0.001 µg/mL aspartame. These results indicate that the TUNEL signal profiles are concordant with the DNA electrophoresis photographs of cells treated with aspartame (Fig. 2) and that the resultant DNA damage is dependent on the degree of apoptosis.

3.3. *Analysis of expression of caspase mRNAs by RT-PCR*

To investigate the mechanism of aspartame-induced apoptosis, the changes of apoptotic factors in PC12 cells treated with aspartame were measured. The expressions of caspase 8 and 9 mRNA in the PC12 cells that were cultured in the serum media containing 0–8 µg/mL aspartame for 72 h were detected by RT-PCR (Fig. 4).

As shown in Fig. 4A, caspase 8 mRNA increased significantly in the PC12 cells cultured in the serum media with $>1 \mu\text{g/mL}$ aspartame as compared to that in the controls. Caspase 9 mRNA also increased significantly in the cells exposed to $>0.01 \mu\text{g/mL}$ aspartame (Fig. 4B).

These results indicated that aspartame induced apoptosis mainly via mitochondrial pathway

3.4. *Expressions of cytochrome c and AIF detected by western blotting*

To investigate whether cytochrome c and AIF are involved in aspartame-induced apoptosis, western blot analyses of the lysis of PC12 cells cultured in serum medium containing aspartame were performed using antibodies against cytochrome c or AIF (Fig. 5).

Apoptosis is induced by the release of cytochrome c from mitochondria into the cytosol (Liu et al., 1996). As shown in Fig. 5A, after treatment with $0.001\text{--}1 \mu\text{g/mL}$ aspartame, the cytochrome c contents in the cytosol of PC12 cells increased significantly as compared to that in the control cells.

As shown in Fig. 5B, the AIF contents were slightly greater in the cells cultured in media containing $0.001\text{--}1 \mu\text{g/mL}$ aspartame than that in the control cells; however, the difference was not significant. These results indicate that apoptosis induced by aspartame mainly depends upon the releasing of cytochrome c.

4. Discussion

The present results confirm that aspartame induces apoptosis in PC12 cells. This finding is corroborated by the results regarding DNA electrophoresis (Fig. 2) and TUNEL

signals (Fig. 3). At first, release of cytochrome c from the mitochondria into cytoplasm (Fig. 5) induces expression of caspase 9 (Fig. 4B). Then increases of the expression triggered the apoptosis. Of course, expression of other caspase (caspase 8) was also increased (Fig. 4A). The aspartame may give multiple effects on the cells.

Aspartame is widely used as an artificial sweetener in low-calorie foods and low-calorie soft drinks. Although many tests on the safety and toxicity of aspartame have been performed, the results are conflicting. The toxicity of a substance is based on the premise that toxicity increases in a dose-dependent manner. Thus, it is not surprising that markedly high doses of aspartame induce deleterious effects in sensitive animal species. The critical question is whether aspartame is potentially harmful at common or abuse usage levels (Stegink et al., 1988). The first evidence of a carcinogenetic effect of aspartame in an animal model was reported by Soffritti et al. (2006). The long-term carcinogenicity of aspartame was demonstrated using a bioassay at a dose close to the human ADI (40 mg/[kg bw·day]). Recently, Abdel-Salam et al. (2012) reported that the administration of aspartame alone or in the presence of a mild systemic inflammatory response increases oxidative stress and inflammation in the brain. If aspartame is related to the production of oxidative stress, it may have the potential to induce apoptosis.

In this study, to evaluate the physiological effects of trace amounts (0–8 µg/mL) of aspartame on cells, the effects of aspartame on apoptosis were investigated using a PC12 cell system. As shown in Fig. 1, aspartame itself induced cytotoxicity. In addition, >1 ng/mL aspartame induced DNA fragmentation due to apoptosis in the PC12 cells (Figs. 2 and 3). The present results show that aspartame itself induced apoptosis in PC12 cells. Some chemical substances enhance apoptosis induced by serum deprivation in PC12 cells (Aoki et al., 2004). In addition, Kurosu and Gunji (2007) reported that

aspartame concentration in beverage was 40-790 µg/ml. The aspartame concentration which induced DNA fragmentation in this study was about 1/40,000 of beverage. Of course, precise toxicity has still been unclear, because there is a possibility that aspartame might induce apoptosis in undifferentiated cells having malignancy.

Apoptosis can be triggered by signals arising from the activation of death receptor-mediated (extrinsic) or mitochondrial-mediated (intrinsic) signaling pathways. Extrinsic apoptotic signaling involves the activation of cell surface death receptors belonging to the protein family of tumor necrosis factor receptors (e.g., Fas) (Itoh and Nagata, 1993). The binding of Fas receptor with its cognate ligand, FasL, can result in activation of caspase 8, activating downstream effector caspases (e.g., caspases 3, 6, and 7), resulting in apoptosis (Boldin et al., 1995; Chinnaiyan et al., 1995; Varfolomeev et al., 1998). On the other hand, the intrinsic apoptotic pathway is regulated by members of the Bcl-2 protein family, which can be divided into anti-apoptotic (e.g., Bcl-2) and pro-apoptotic (e.g., Bax and Bad) proteins. Bcl-2 family members play a central role in regulating changes in mitochondrial membrane permeability (Adams and Cory, 1998; Gross et al., 1998; Shimizu et al., 1999; Chipuk and Green, 2008). After an apoptotic stimulus, the pro-apoptotic proteins trigger the release of cytochrome c and AIF from mitochondria (Green, D. R. and Reed, 1999). Cytochrome c accelerates the activation of caspase 9, initiating a downstream caspase cascade, which ultimately leads to cell death. AIF induces apoptosis via a caspase-independent pathway when cells experience serious oxidative stress.

The present results confirm that aspartame induces apoptosis in PC12 cells. To determine the apoptotic pathway initiated by aspartame treatment, the changes in caspase 8 (the initiator caspase associated with death receptor apoptotic signaling) and

caspase 9 (the initiator caspase associated with mitochondrial apoptotic signaling) in PC12 cells treated with aspartame were analyzed by RT-PCR. As shown in Fig. 4A, the expression level of caspase 8 increased significantly in PC12 cells treated with >1 $\mu\text{g}/\text{mL}$ aspartame. In addition, the expression level of caspase 9 increased significantly in PC12 cells treated with 0.01 – 1 $\mu\text{g}/\text{mL}$ aspartame (Fig. 4B). The results indicate that aspartame induces apoptosis mainly via mitochondrial pathways. Furthermore, one possibility is still remaining that high concentration aspartame may induce via death receptor pathways. It is unclear why there are 2 aspartame-induced apoptotic pathways. However, it has been shown that apoptosis induced by cobalt chloride in PC12 cells was mediated by mitochondria as well as death receptor pathways (Jung and Kim, 2004). Caspase 8, which activates the pro-apoptotic Bid protein, is the link between the extrinsic apoptotic pathway initiated by death receptors and the intrinsic pathway mediated by mitochondria (Li et al., 1998). Truncated Bid is inserted into the mitochondrial outer membrane, releasing cytochrome c and resulting in the activation of caspase 9 (Hengartner, 2000). Future studies on the interaction between the death receptor and mitochondrial mediated pathways are needed to clarify the detailed mechanism of aspartame-induced apoptosis.

Moreover, to confirm whether apoptosis induced by low concentration of aspartame, dependent on caspase 9, was caused by the increased cytochrome c (caspase-dependent cell death effector) or AIF (caspase-independent cell death effector) levels, the cellular contents of both were measured using western blot method. As shown in Fig. 5, cytochrome c expression was significantly greater in PC12 cells after treatment with <1 $\mu\text{g}/\text{mL}$ aspartame than that in the controls. In contrast, there was no significant difference in AIF expression between cells treated with and without aspartame. These

results indicate that low concentrations of aspartame that induce apoptosis in the caspase-dependent pathway are involved in the release of cytochrome c from mitochondria.

The balance between pro- and anti-apoptotic proteins of the Bcl-2 family plays a crucial role in the release of cytochrome c. In response to DNA damage, p53 is activated and regulates gene expression (Heimbrook and Oliff, 1998); it induces the activation of Bax and Bad as well as the suppression of Bcl-2 (Schuler and Green, 2001). In addition, aspartame induces reactive oxygen species (ROS) production in mammalian cells (Alleva et al., 2011). It is hypothesized the synthesis of ROS, which is catalyzed by aspartame, damages the DNA in PC12 cells. These ROS then trigger the transactivation of p53, resulting in the activation of Bax and Bad. In this case, the activated pro-apoptotic proteins induce the release of cytochrome c from mitochondria.

Apoptosis caused by DNA damage is harmful to living organisms. If unrepaired, DNA damage in injured cells leads to errors during DNA synthesis, resulting in some mutations that can give rise to cancer. Therefore, individuals with an inherited impairment in DNA repair capability often have an increased risk of cancer (Bernstein et al., 2011). Sato et al. (2011) report that curcumin induces apoptosis and suppresses cancer initiator signals.

In conclusion, aspartame induces apoptosis in PC12 cells. This induction is thought to occur via the increased release of cytochrome c into the cytosol via the mitochondrial pathway. In addition, it has possibility that high concentration of aspartame may induce apoptosis via the death receptor pathway. Of course to clarify the validity of toxic mechanism and relationship of carcinogenicity for aspartame, further investigation using other cultured cells or experimental animals should be investigated in detail on

apoptotic stage.

“Conflict of Interest”

The authors declare that there are no conflicts of interest.

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Figure Legends

Fig. 1-Cell viability (A) and LDH activities in the serum media (B) of PC12 cells cultured in media containing 0–8 µg/mL aspartame for 72 h

In (A), the values were calculated using the following formula: (non-staining cell number ÷ total cell number) × 100. In (B), LDH activities are expressed as the activity relative to that of the serum medium without aspartame. PC12 cells cultured in medium without aspartame for 72 h were used as controls. Error bars indicate SEM (n = 3). *P < 0.05, **P < 0.01 vs. control.

Fig. 2-Agarose gel electrophoresis of genomic DNA extracted from PC12 cells cultured in media for 72 h after treatment with 0–8 µg/mL aspartame

DNA from PC12 cells incubated in medium without aspartame for 72 h were used as controls. M: λDNA digested with *HindIII* as DNA size marker

Fig. 3-The DNA fragmentation in PC12 cells cultured in media with 0–8 µg/mL aspartame for 72 h was quantified using the TUNEL method

TUNEL signals are expressed relative to that of the control cells. Error bars indicate SEM (n = 4), *P < 0.05, **P < 0.01 vs. controls.

Fig. 4-Expressions of caspase 8 (A) and caspase 9 (B) mRNA in PC12 cells exposed to 0–8 µg/mL aspartame for 72 h quantified by RT-PCR

Expression levels of mRNA were calculated as n mol / n mol β-actin (as an internal control) and are expressed relative to that of the control cells. Error bars indicate

SEM (n = 4) for caspase 8 and (n = 5) for caspase 9. *P < 0.05 vs. controls.

Fig. 5-Representative western blot analysis of homogenates of PC12 cells treated with 0–1 µg/mL aspartame for 72 h against cytochrome c (A) and AIF (B) antibodies

In each upper panel, a typical electrophoresis pattern is present. The mean staining intensities relative to that of the control (set to 100) are shown. The data represent the SEM (n = 4) for cytochrome c and (n = 5) for AIF. *P < 0.05 vs. controls.