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Cigarette Smoke Extract Induces Bronchial Epithelial Cell Death by Caspase 1-dependent Mechanism

흡연 추출물에 의해 유도되는 기관지
상피 세포의 caspase 1 의존적 사멸

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Cigarette Smoke Extract Induces Bronchial Epithelial Cell Death by Caspase 1-dependent Mechanism

by

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ABSTRACT

Introduction: Cigarette smoke, which contains over 4,000 chemical compounds, could affect cell death in various manners. Although several mechanisms including apoptosis and necrosis were proven in cell deaths caused by cigarette smoke, pyroptosis which is caspase 1-dependent cell death was not demonstrated yet. The aim of this study is to prove pyroptosis of bronchial epithelial cells triggered by cigarette smoke.

Methods and Results: BEAS-2B cells were treated with different concentration of cigarette smoke extract (CSE). After CSE treatment for 12, 24 and 48 hours, MTT assay was performed to measure cell viability according to CSE concentration. When cells were treated for 24 hours with 7.5% CSE, cell viability began to decline significantly. However, cell survival rate was increased when 2.5% CSE was treated.

As pyroptosis is initiated by caspase 1 activation, caspase 1 expression and activity were assessed. Increased caspase 1 expression by CSE was confirmed by western blotting. Caspase 1 activity measured by calorimetric kit was also increased with CSE treatment. Because the key feature of pyroptosis is pore formation, ethidium bromide (EtBr) uptake was measured. Cell death following pore formation was evaluated with MTT assay and LDH release assay. EtBr uptake and cell deaths were increased when CSE was treated. However, when caspase 1 inhibitor (Z-YVAD-FMK 10μM) was treated together, EtBr uptake and cell deaths (MTT assay, LDH release assay) were significantly decreased. Moreover, flow cytometry result showed increased

size of CSE treated cells suggesting pore formation and cell swelling.

However, as cell death could not be wholly explained by pyroptosis, the hallmarks of apoptosis and autophagy were measured. CSE activated caspase 3 and this mediated PARP cleavage. Moreover, Bcl-xL level was decreased by CSE treatment. Flow cytometry using fluorescein-conjugated Annexin V also showed the increased percentage of apoptotic cells by CSE. Finally, cell viability using MTT assay and LDH release assay under CSE treatment was decreased when caspase 3 inhibitor (Z-DEVD-FMK 10μM) was added. The hallmarks of autophagy (Beclin 1 and LC-3B) were also activated by CSE. In addition to the hallmarks of apoptosis and autophagy, senescence markers were also evaluated. p21 and p27 were activated by CSE and these activation could explain the reason why cell survival rather than cell death occurred in a certain concentration of CSE.

Conclusions: Cigarette smoke could induce caspase 1-dependent cell death (pyroptosis) of bronchial epithelial cells. Moreover, other cell death mechanisms such as apoptosis or autophagy as well as pyroptosis could contribute to the cell death by cigarette smoke.

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Keywords: Pyroptosis, caspase 1, cigarette smoke extract, bronchial epithelial cell

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INTRODUCTION

Cigarette smoke, which contains over 4,000 chemical compounds, is well known risk factor for several respiratory disease(1). The chemical mechanisms of lung cancer by cigarette smoke had been elucidated through previous study(2). Also, the mechanisms of cigarette smoke-induced chronic obstructive lung disease (COPD) had been suggested(3, 4). However, the impact of cigarette smoke extract (CSE) on cell death has controversy yet.

Cigarette smoke-induced DNA damage in bronchial epithelial cell was not lethal and CSE did not induce cell death under a certain condition(5). Moreover, CSE induced senescence in alveolar epithelial cells characterized by overexpression of p21(6). Overexpression of p21 could influence cell proliferation and cell survival(7). However, CSE could also have cytotoxic effect by inducing apoptosis and necrosis(8-10). As CSE consisted of over 4,000 chemical compounds, various mechanisms could be involved in cell death by CSE.

Pyroptosis, which is a programmed cell death distinguished from apoptosis, is currently recognized as one of the cell death mechanisms in innate immune cells(11). When caspase 1 is activated by several infectious stimuli, caspase 1-dependent cell death, so called pyroptosis, occurs in immune cells(11). Although the exact signaling pathway of pyroptosis has not been demonstrated, pore formation on cellular membrane and DNA cleavage are the key features of pyroptosis(11-13).

The relationship between CSE and pyroptosis has not been proven yet.

Furthermore, pyroptosis that is known to occur in immune cells has not been observed in epithelial cells(11). However, caspase 1 could be up-regulated by CSE and bronchial epithelial cells could act as innate immune cells(14, 15). Exposure to cigarette smoke led to the activation of inflammasome and caspase 1 activation was derived(14, 16). Also, exposure to cigarette smoke induced the release of pro-inflammatory cytokine from bronchial epithelial cells(17). Thus the chance of pyroptosis in bronchial epithelial cells by CSE could be addressed. Here, we report the pyroptosis of bronchial epithelial cells triggered by CSE.

METHODS

Cell Culture

BEAS-2B cells were maintained in keratinocyte serum free medium (Life technology, #10744-019) containing supplemented growth factor, penicillin 20U/ml and streptomycin 20μg/ml. Cells were grown and maintained in 100 mm^2 dishes at 37°c in a 5% CO_2 humidified incubator. The cells were treated with various concentration of cigarette smoke extraction according to the designated time schedule.

Preparation of Cigarette Smoke Extract

Cigarette smoke extract (CSE) was prepared by method based on previous report(8). Briefly, 20 unfiltered cigarettes, each containing 0.65 mg of nicotine and 6.5 mg of tar according to the manufacturer's report, were smoked consecutively through a chest tube bottle with a constant vacuum flow. Vacuum flow was generated under 60 psi by vacuum generator (GAST manufacturing). The smoke was bubbled through 60ml of keratinocyte serum free medium. Then, the obtained CSE was filtered through a 0.22-μm pore filter. The CSE was prepared just before each experiment. CSE treatments were performed according to the designated concentration and time schedule.

Reagents

CytoTox-ONE Homogenous Membrane Integrity Assay (#G7890) was purchased from Promega (Madison, USA). Caspase-1 Calorimetric Assay Kit

(#BF14100) was supplied from R&D systems (Minneapolis, USA). Caspase-1 inhibitor (Z-YVAD-FMK, #1012-20C) was obtained from Biovision (Milpitas, USA) and caspase 3 inhibitor (Z-DEVD-FMK, #SC3075) was purchased from Santa Cruz (Santa Cruz, USA). Rabbit polyclonal anti-p27(#SC-528), anti-p21 (#SC-397), anti-Beclin-1 (#SC-11427), anti-cIAP-1(#SC-7943), anti-cIAP-2 (#SC-7944), anti-Bcl-xL(#SC-7195) and Mouse monoclonal anti-tubulin (#SC-5286) were purchased from Santa Cruz (Santa Cruz, USA). Rabbit polyclonal anti-LC-3B (#CS-2775), anti-caspase-1 (#CS-2225), anti-casapse-3 (#CS-9661) and anti-PARP (#CS-9542) was obtained from Cell Signaling (Beverly, USA). Rabbit polyclonal anti-LAMP-2A (#ab18528) was purchased from Abcam (Austin, USA). Goat anti-rabbit secondary antibody (#NCI1460KR) and Goat anti-mouse secondary antibody (#NCI1430KR) were obtained from Thermo Scientific (Fremont, USA).

Cell Viability Test

Cell viability test was measured by the MTT (3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded on 35mm^2 dishes with 6x10^5 cells in 1ml media per well. After 48 hours stabilization of the cells, the cells were treated with 25µg/ml, 50µg/ml, 75µg/ml, 100µg/ml, 150µg/ml, and 200µ/ml of CSE for 24 hours. Furthermore, the cells were exposure to 50µg/ml, 100µg/ml, 150µg/ml, and 200µ/ml of CSE for 12 hours and 48 hours respectively. After the treatment of CSE, 100µl of MTT solution (100µl/ml) was added to each plate and the cells were incubated for 30 minutes at 37°c in a 5% CO_2 humidified incubator. Then, the cells were

treated with 300µl of DMSO solution and the absorbance at 550nm was read by the microplate spectrophotometry. The viability of each plate was expressed as the percentage of control group assumed to be 100%.

Cell viability test was also measured by lactate dehydrogenase (LDH)-release assay. Promega Kit (#G7890) was used. This kit is based on the fact that dying cells lose the integrity of membrane and release cytoplasmic enzyme including LDH. 50µl of medium in each cell plate was seeded at 96-well plates and 50µl of CytoTox-ONE reagent was added. After 10 minutes of incubation at room temperature, 25µl of stop solution was added to terminate the reaction. The fluorescence was assessed immediately at excitation of 550nm and emission at 590nm. The degree of cell deaths was expressed as the rate of LDH release compared with that of control cells.

Western Blotting

$1.2x10^6$ cells were treated with 10µg/ml, 25µg/ml, 50µg/ml, 75µg/ml, 100µg/ml, 150µg/ml, and 200µ/ml of CSE for 24 hours at 60 mm^2 dishes. At the end of treatment, cells were gently washed with PBS twice and 70 µl of cell lysis buffer solution was added to each dish. Each sample containing 50 µg protein was solubilized with 8.75µ LDS samples loading buffer (Life Technology,#NP0007) and 3.5 µl reducing agent (Life Technology ,#NP0004). Samples were loaded per lane and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk/PBS with 0.1% Tween for 30 minutes before incubation at 4°c with primary antibodies diluted to 1:1,000 in 5% skim milk/PBS with 0.1% Tween. After overnight incubation, the

membranes were washed with TBS-T three times for 30 minutes and incubated with secondary antibodies diluted to 1:2,000 in 5% skim milk/PBS with 0.1% Tween. The membranes were developed using ECL kit.

Flow Cytometry

Light scattering properties of the cells were measured by flow cytometer after CSE treatment. Forward (FSC-H) and side (SSC-H) scatter were analyzed and a density plot was constructed with FSC plotted along x-axis and SSC plotted along y-axis.

Fluorescein-conjugated Annexin V was used to quantitatively determine the proportion of cells undergoing apoptosis. The percentage of apoptotic cells was analyzed by flow cytometry.

Analysis of Pyroptosis

The expression of caspase-1 was assessed using Western blotting after treatment with 10μg/ml, 25μg/ml, 50μg/ml, 75μg/ml, 100μg/ml, 150μg/ml, and 200μ/ml of CSE for 24 hours. Caspase-1 activity was also evaluated using caspase-1 calorimetric activity assay kit. After co-treatment of 10μM of caspase-1 inhibitor and CSE with various concentration as mentioned above for 24 hours, MTT assay and LDH assay were performed to evaluate cell viability. To measure the degree of pore formation, the cells on plates were washed with PBS and stained with 10μg/ml of Hoechst and 25μg/ml of ethidium bromide (EtBr) for 3 minutes. Then, the cells were observed with fluorescence microscopy at 450nm and 590nm. Cells that were treated with 1%

Triton X-100 were 100% lysis control.

Statistical Analysis

Data are represented as mean and standard deviation. A student t-test and chi-square test were used to compare the difference between control groups and paired controls. A value of $p<0.05$ was considered to indicate significance. All statistical analyses were performed using SPSS software, version 21.0 (Chicago, USA).

RESULTS

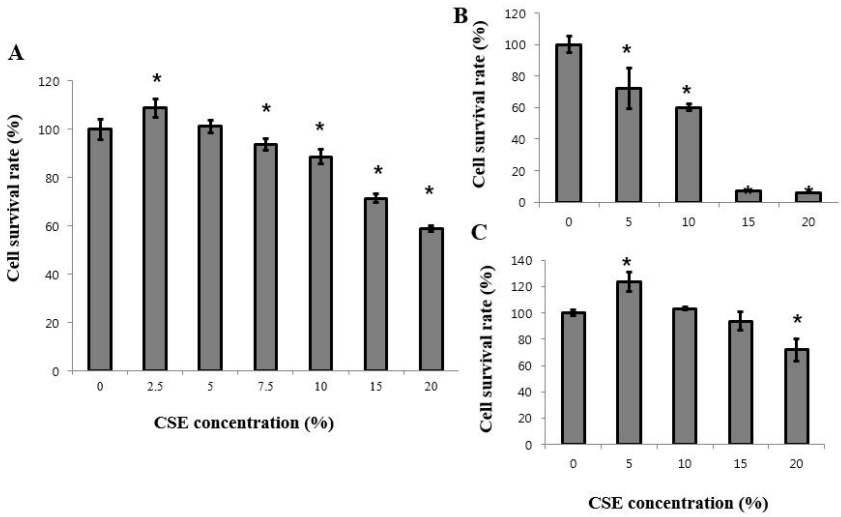
CSE stimulates death of bronchial epithelial cell

To investigate the impact of CSE on cell death, BEAS-2B cells were treated with various concentration of CSE. After treatment for 24 hours, MTT assay was performed. With CSE treatment over 7.5% concentration, cell deaths happened in dose-dependent manner (Figure 1a). With treatment of CSE for 48 hours, cell deaths began to start at 5% CSE and only 6.1% of BEAS-2B cells survived at 20% CSE. However, when stimulated for 12 hours, the degree of cell deaths was decreased compared those with treatment for 24 hours or 48 hours. In fact, cell survival rather than cell death was observed at 5% of CSE (Figure 1c). These results suggested that various mechanisms were involved in BEAS-2B cells deaths caused by CSE.

Caspase 1 was expressed in BEAS-2B cells after exposure of CSE

To prove pyroptosis in BEAS-2B cells triggered by CSE, we tried to verify the expression of caspase 1 after exposure of CSE. As shown in figure 2a, caspase 1 was up-regulated after treatment of CSE for 24 hours in western blotting. Furthermore, the activity of caspase 1 was measured using caspase 1 calorimetric assay kit. The activity of caspase 1 was increased under the stimulation with 5%, 7.5% and 10% CSE for 24 hours and the activity showed the highest value with 10% CSE for 24 hours (Figure 2b). However, exposure more than 10% CSE did not affect the activity of caspase 1 in BEAS-2B cells.

Figure 1. Effect of CSE on the viability of BEAS-2B cells was assessed using MTT assay.



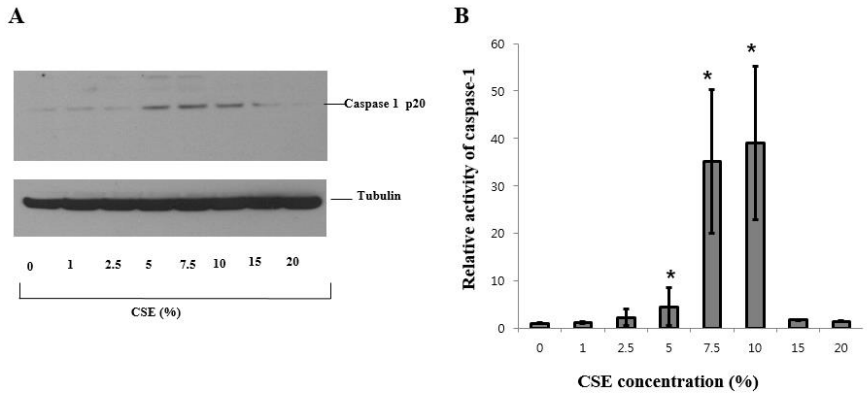
A. Cell survival rate was increased when 2.5% CSE was treated for 24 hours. However cell viability was significantly decreased from 7.5% CSE.

B. When cells were treated for 48 hours, cell viability decreased from 5% CSE.

C. When cells were treated for 12hours, cell survival rate was increased with 5% CSE while cell death was observed with 20% CSE.

Data are represented as the mean ± SD. An asterisk indicates $p<0.05$ compared with cells without CSE treatment.

Figure 2. CSE increased the expression of caspase 1 and caspase 1 activity



A. Active form of casapse 1 (p20) was detected by western blotting when BEAS-2B cells were treated with 5-10% CSE for 24 hours.

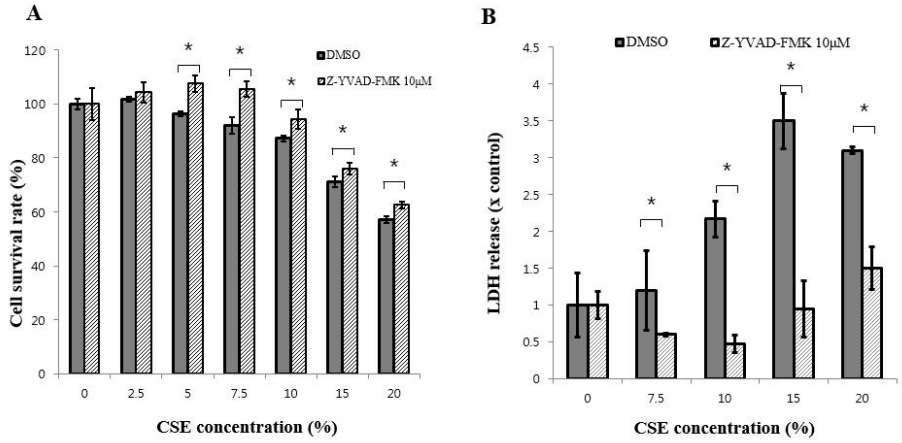
B. Caspase 1 calorimetric assay kit was used to measure the activity of caspase 1 according to the CSE concentration for 24 hours. According to manufacturer's instruction, activity was quantified with using microplate read at 405 nm. Caspase 1 activity was increased when CSE concentration was 5%, 7.5% and 10%.

Data are represented as the mean ± SD. An asterisk indicates $p<0.05$ compared with cells without CSE treatment.

Caspase 1 inhibitor suppressed cell death triggered by CSE

After verifying the expression of caspase 1 by CSE, we tried to prove the relationship between caspase 1 and cell death. After exposure of CSE for 24 hours, MTT assay was repeatedly preformed. As previously mentioned, with increment of CSE concentration, more cell deaths were observed. However, with co-treatment of caspase 1 inhibitor (Z-YVAD-FMK 10μM), cell deaths were decreased significantly (Figure 3a). Because the degree of LDH release also reflected the state of cell death including pyroptosis(13, 18), we confirmed caspase 1 dependent cell death using LDH release assay. As MTT assay demonstrated, CSE induced more LDH release and this tendency disappeared when caspase 1 inhibitor was co-treated with CSE (Figure 3b). However, caspase 1 inhibitor did not prevent cell deaths wholly although caspase 1 inhibitor reduced the degree of cell death by CSE. This result suggested that pyroptosis was involved in cell death raised by cigarette smoke. However, pyroptosis could not be used to account for cell death entirely and other cell deaths mechanisms such as apoptosis could be contributed to cell death by CSE. Before caspase 1 inhibitor was used, the effect of inhibitory function was confirmed using caspase 1 activity kit (Figure 4).

Figure 3. Caspase 1 inhibitor prohibited cell death triggered by CSE

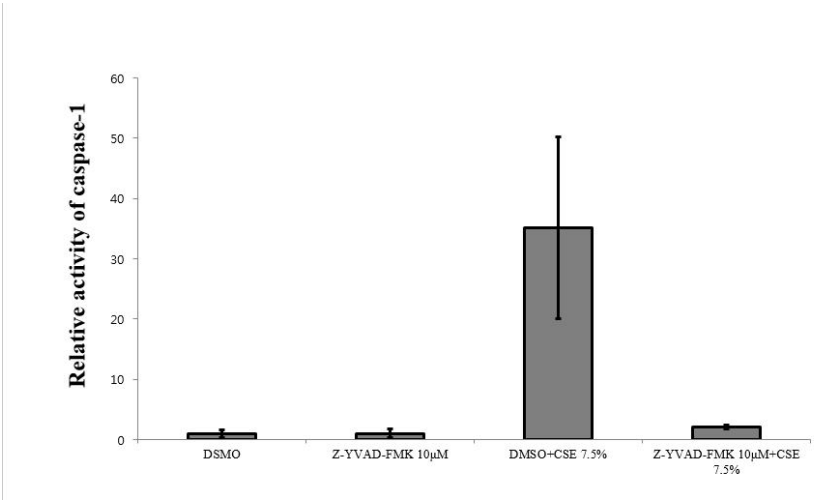


A. Viability of BEAS-2B cells assessed by MTT assay was increased when caspase 1 inhibitor (Z-YVAD-FMK, 10μM) was added to CSE for 24 hours compared with cells that were treated with CSE and DMSO (Vehicle).

B. LDH release assay results also showed the increased viability of BEAS-2B cells when caspase 1 inhibitor was added to CSE.

Data are represented as the mean ± SD. An asterisk indicates $p<0.05$ between DMSO treated cells and caspase 1 inhibitor treated cells.

Figure 4. The effect of caspase 1 inhibitor (Z-YVAD-FMK 10μM) was confirmed using caspase 1 calorimetric assay kit



The effect of caspase 1 inhibitor was evaluated using caspase 1 calorimetric assay kit. When BEAS-2B cells were treated with CSE and Z-YVAD-FMK 10μM for 24 hours, the activity of caspase 1 was significantly decreased. Data are represented as the mean ± SD.

Caspase 1 mediated ethidium bromide uptake by BEAS-2B cells

Pore formation mediated by caspase 1 and resultant osmotic swelling of cells are key features of pyroptosis. Therefore, ethidium bromide (EtBr) staining which did not stain cells with intact membranes has been used to prove pyroptosis in previous studies(13, 19-21). In previous studies, pyroptotic cells were stained with EtBr while viable cells were not stained. Likewise, apoptotic cells were not stained with EtBr as apoptotic cells maintained intact plasma membrane(13).

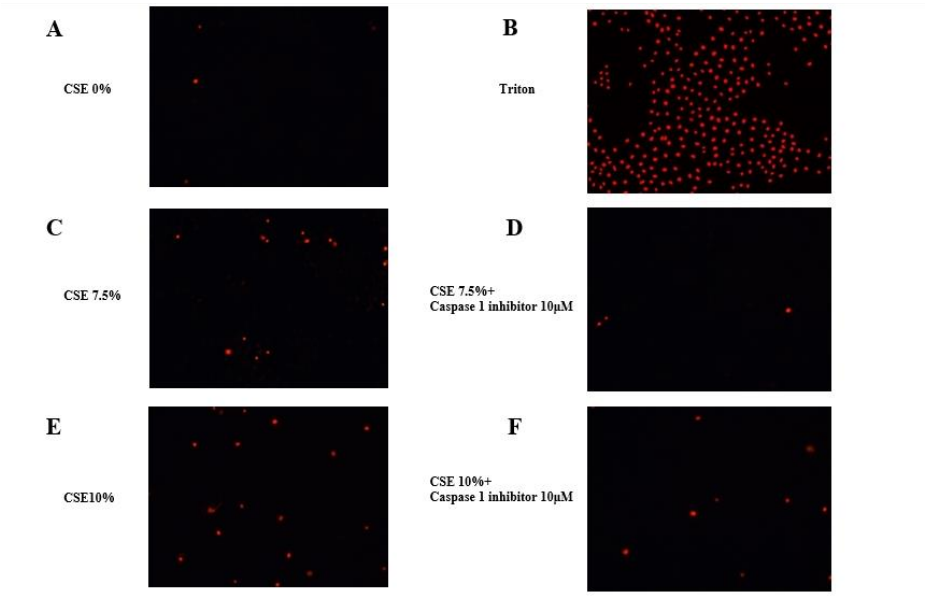
In this study, after treatment of CSE for 24 hours, EtBr dye and Hoechst dye were stained to BEAS-2B cells. As shown in figure 5, CSE mediated EtBr uptake by BEAS-2B cells in a dose dependent manner. However, when caspase 1 inhibitor was treated, EtBr uptake by BEAS-2B cells was decreased. We also counted the number of cells that were stained with EtBr with the aid of two independent raters. The proportion of cells stained by EtBr was increased as the concentration of CSE increased. However, with co-treatment of caspase 1 inhibitor, the proportion of EtBr stained cells was significantly decreased under 5%, 10% and 15% CSE (table 1). These results suggested that caspase 1 mediated EtBr uptake and supplemented the evidence of pyroptosis triggered by CSE.

CSE induced size increase of BEAS-2B cells

Flow cytometry was performed to assess cell size and cell granularity measuring the forward scattering (FSC-H) and side scattering (SSC-H) parameters. In fact, cells dying by pytoptosis undergo a measurable size

increase while cell shrinkage develops in an early apoptosis(11, 22). Therefore, the increase of cell size could suggest the evidence of pyroptosis. The FSC-H and SSC-H of CSE treated cells are shown in figure 6. When BEAS-2B cells were treated with 20% CSE for 24 hours, the right side shift of FSC-H was observed and this increase of cell size supported the evidence of pyroptosis triggered by CSE.

Figure 5. CSE increased EtBr uptake while caspase 1 inhibitor (Z-YVAD-FMK 10μM) prevented uptake.



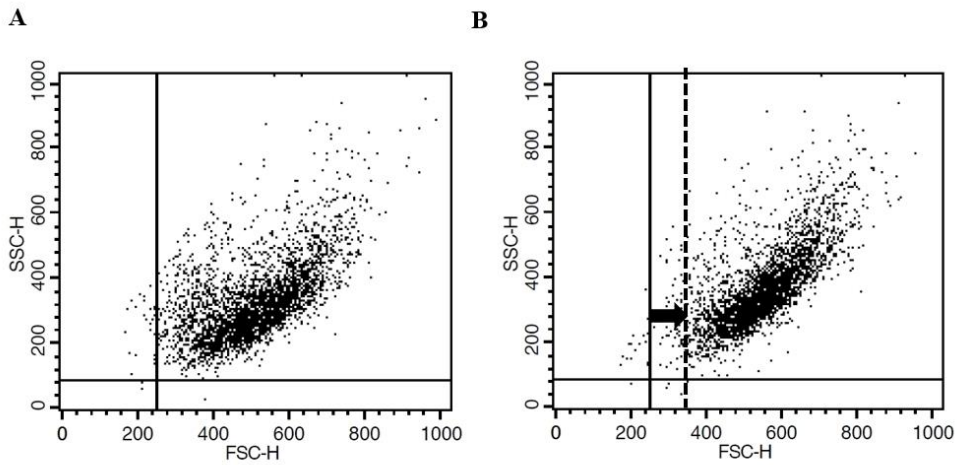
After treatment of CSE for 24 hours, cells were stained with 25μg/ml EtBr for 3minutes. Then, cells were visualized by fluorescence microscopy. **(A)** CSE 0%, **(B)** 1% Triton as positive control, **(C)** CSE 7.5%, **(D)** CSE 7.5% with caspase 1 inhibitor 10μM, **(E)** CSE 10%, and **(F)** CSE 10% and caspase 1 inhibitor 10μM. Representative images from three experiments are shown.

Table 1. The percentage of EtBr uptake cells when BEAS-2B cells were exposed to CSE with or without caspase 1 inhibitor

CSE concentration	DMSO	Z-YVAD-FMK 10μM	p-value
0%	0.8%	0.4%	0.145
5%	3.2%	1.8%	0.009
10%	4.4%	2.8%	0.001
15%	6.0%	2.5%	<0.001
20%	10.3%	10.4%	0.875

Independent rater observed through fluorescence microscopy and decided six representative images of each plate. Then, two other independent raters counted the number of EtBr. uptake cells.

Figure 6. CSE increased the size of BEAS-2B cells



A. Forward (FSC-H) and side scattering (SSC-H) data from FACS analysis of BEAS-2B cell that were treated without CSE for 24 hours are shown.

B. The overall increase of forward scattering was observed when BEAS-2B cells were treated with 20% CSE.

CSE induced the hallmarks of apoptosis

As mentioned above, pyroptosis could not entirely explain the cell deaths by CSE and this suggested the existence of other cell death mechanism. Thus, the hallmarks of apoptosis were investigated.

Cellular inhibitors of apoptosis protein (cIAP-1, cIAP-2) were not affected by the exposure of CSE. However, Bcl-xL was decreased when BEAS-2B cells were exposed to CSE. As a result, caspase 3 was activated by CSE and this mediated PARP cleavage (Figure 7).

CSE increased the percentage of apoptotic cell

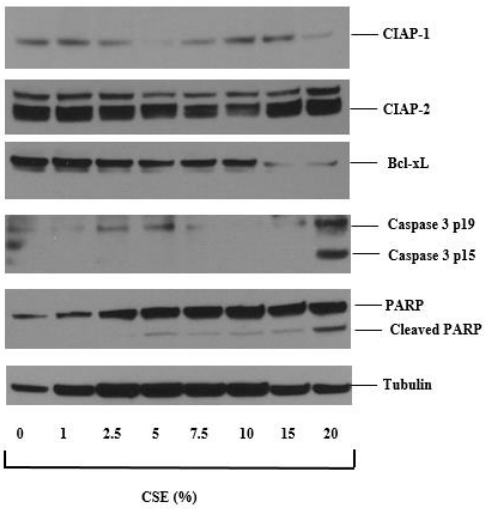
To quantitatively assess the degree of apoptosis, flow cytometry using fluorescein-conjugated Annexin V was performed. When BEAS-2B cells were incubated for 24 hours without the stimulation of CSE, the percentage of apoptotic cells was 4.9%. However, under 20% CSE, the percentage of these cells was increased up to 15.6% (Figure 8). Therefore, CSE could induce apoptotic death of BEAS-2B cells.

Caspase 3 inhibitor prevented cell death by CSE

As apoptosis was mediated by the activation of caspase 3, caspase 3 inhibitor (Z-DEVD-FMK) was treated to confirm the apoptotic cell deaths. When 10% CSE and 10 μM caspase 3 inhibitor were co-treated, cell viability using MTT assay was increased up to 11.6% compared with that with 10% CSE alone. When LDH release assay was performed, the amount of released LDH was also decreased as caspase 3 inhibitor was treated.

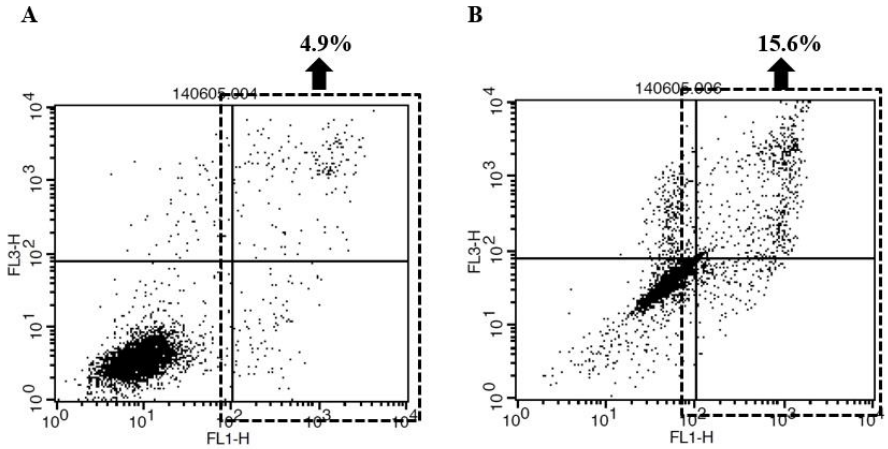
When caspase 1 inhibitor(10 μM) and caspase 3 inhibitor (10 μM) were added together to 10% CSE treated cells, cell viability was increased up to 12.0% and the amount of released LDH was decreased by one third. However, these values were similar to those of cells which were treated with caspase 3 inhibitor alone under 10% CSE (MTT assay; *p*-value 0.376, LDH release assay; *p*-value 0.513) (Figure 9).

Figure 7. Western blotting of hallmarks of apoptosis according to CSE concentration



The expression of CIAP-1/2 was not affected by CSE. Bcl-xL was decreased and caspase 3 was activated when CSE was added to BEAS-2B cells for 24 hours. This activation of caspase 3 mediated PARP cleavage.

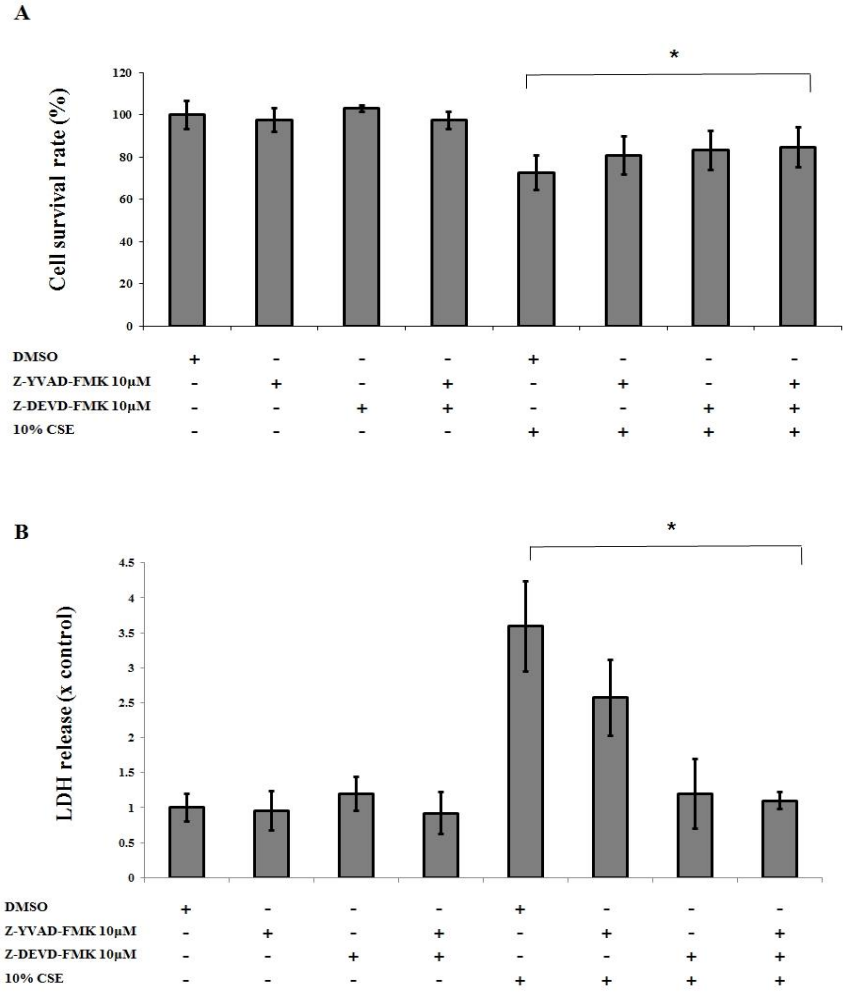
Figure 8. CSE increased the percentage of apoptotic cells



A. BEAS-2B cells were treated without CSE for 24 hours and flow cytometry using fluorescein-conjugated Annexin V was performed**.** The percentage of apoptotic cells was 4.9%.

B. When BEAS-2B cells were treated with 20% CSE for 24 hours, the percentage of apoptotic cells was 15.6%

Figure 9. Caspase 3 inhibitor decreased cell death by CSE



10% CSE with DMSO (vehicle), 10% CSE with caspase 3 inhibitor (Z-DEVD-FMK 10 μM), 10% CSE with caspase 1 inhibitor (Z-YVAD-FMK 10 μM), and 10% CSE with caspase 1 inhibitor/caspase 3 inhibitor were added to BEAS-2B cells for 24 hours.

A. In MTT assay, caspase 3 inhibitor increased cell viability up to 11.6%

B. Caspase 3 inhibitor decreased the degree of LDH release.

The co-treatment with caspase 1 inhibitor did not showed the synergistic effect coordinated with caspase 3 inhibitor in terms of cell viability when using MTT assay (p=0.376) and LDH release assay (p=0.513).

Data are represented as the mean ± SD. An asterisk indicates p<0.05

CSE induced the hallmarks of autophagy

As another cell death mechanism, autophagy dependent cell death could be suggested. Autophagy induced by cigarette smoke and autophagy dependent cell death have been elucidated in previous studies(23, 24). As shown in figure 10, Beclin 1 and LC 3B, which are hallmarks of autophagy, were activated by CSE. Thus, CSE induced autophagy of BEAS-2B cells. However, LAMP-2A expression was not changed irrespective of CSE concentration. This result suggested that chaperone mediated autophagy which are mediated by LAMP-2A was not associated with autophagy induced by CSE(25, 26).

CSE could induce cell survival rather than cell death

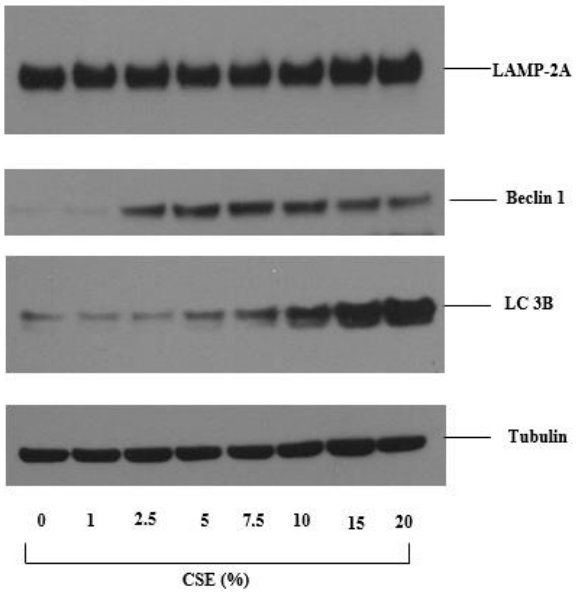
As shown in figure 1a, cell deaths were observed when the concentration of CSE was more than 7.5%. However, cell deaths did not occur under 5% CSE and cell survival rather than cell death was witnessed when BEAS-2B cells were exposed to 2.5% CSE for 24 hours. This result suggested that CSE could induce cell survival as well as cell death at the same time. As CSE could induce cellular senescence and this would prohibit apoptotic cell death(5, 27, 28), the hallmarks of cellular senescence were evaluated.

The expression of p53 was not affected by CSE stimulation. However, p21, which is known to prohibit p53-dependent apoptosis and to act as a feedback mechanism to control p53 activity, was activated with CSE treatment in a dose-dependent manner(7, 28, 29).

However, p27 might use different mechanisms to regulate cell death and the

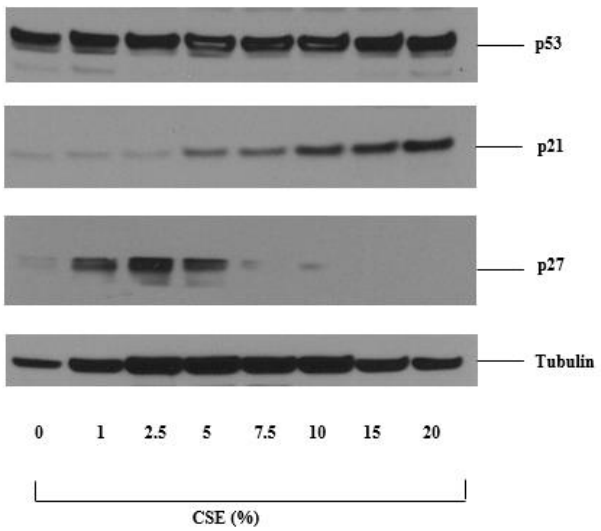
expression pattern of p27 was different from that of p21(29). p27 was activated only when the concentration of CSE was between 1 and 5% (Figure 11).

Figure 10. Western blotting of hallmarks of autophagy according to CSE concentration



Beclin 1 and LC3B in BEAS-2B cells were activated by CSE treatment for 24 hours. Compared with Beclin 1 and LC3B, the expression of LAMP-2A was not affected by CSE.

Figure 11. Western blotting of hallmarks of senescence according to CSE concentration.



p53 expression in BEAS-2B cells was not affected by CSE exposure for 24 hours. p21 activation was increased by CSE in a dose dependent manner. p27 was activated when 1-5% of CSE was treated for 24 hours.

DISCUSSION

This study was aimed to prove pyroptosis of BEAS-2B cells triggered by cigarette smoke. In this study, CSE mediated caspase 1 activation and this mediated pyroptosis of BEAS-2B cells. However, cell death by CSE could not be explained entirely by pyroptosis. Thus, the other mechanisms could be involved in CSE-related cell deaths. We found that apoptosis and senescence occurred when BEAS-2B cells were treated with CSE. Furthermore, we also found the possibility of autophagy dependent cell death.

The major function of caspase 1 has been known as a modulator of inflammatory response. Caspase 1 is activated by inflammasome and activated caspase 1 mediates the maturation of interleukin (IL)-1β and IL -18(30). Therefore, caspase 1 has been considered to be mainly involved in innate immune regulation. However, caspase 1 could affect cell death independently of its pro-inflammatory cytokines processing(31). The cell death mediated by caspase 1 has been recognized currently and has been called as pyroptosis. Early in its discovery, the role of pyropotis was suggested to augment innate immunity and eradicate intracellular pathogens(32). Thus, pyroptosis has been considered to occur only in innate immune cells by infectious stimuli(11). Contrary to this conventional knowledge about pyroptosis, pyroptosis triggered by non-infectious stimuli was observed in non-immune cells. In this study, CSE activated caspase 1 and this mediated pyroptosis of BEAS-2B cells. In fact, the association between CSE and caspase 1 activation has been suggested through previous studies (14,

16). Moreover, bronchial epithelial cell could act as innate immune cell(15). Therefore, the pyroptosis of bronchial epithelial cells induced by CSE could be inferred. However, no studies have proven the pyroptosis by CSE and this is the first study to prove that pyroptosis was one of the cell death mechanisms by cigarette smoke in bronchial epithelial cells.

Until now, the signal pathway of pyroptosis has not been fully elucidated. The upstream pathway above caspase 1 activation included the NLRC3, NLRP3, and AIM2 activation(12). However, the signal pathway following caspase 1 activation is still unknown. Nevertheless, caspase 1-dependent pore formation and cell swelling following pore formation have been thought to be the key features of pyroptosis(11). As a result, many previous studies had proved the generation of pyroptosis with measuring pore formation (13, 19-21). Also, measuring the degree of cell lysis following pore formation was used to prove the cell death by pyroptosis(13, 19). In this study, the pore formation was assessed using EtBr uptake and cell lysis was measured by LDH release assay. Although EtBr uptake and LDH release assay could be used when investigating other cell deaths mechanisms(33, 34), the degree of EtBr uptake and LDH release were decreased when caspase 1 inhibitor (Z-YVAD-FMK, 10μM) was added. Above all, cell death accordance with caspase 1 activity was significantly decreased when caspase 1 inhibitor was added. Therefore, pyroptosis caused by CSE in BEAS-2B cells was reasonably proven in this study.

However, as mentioned in result section, the cell deaths by CSE could not be explained entirely by pyroptosis. In fact, other cell death mechanisms

including apoptosis, necrosis and autophagy dependent cell death had been introduced(8-10, 23, 24). In this study, we found that the hallmarks of apoptosis were increased by CSE. Moreover, the inhibition of caspase 3 activity decreased cell death caused by CSE. Interestingly, the degree of cell death prevention by caspase 3 inhibitor was greater than that by caspase 1 inhibitor. Moreover, co-treatment of caspase 1 inhibitor and caspase 3 inhibitor to BEAS-2B cells under CSE stimulation did not have synergistic effect in terms of cell viability. These results suggested that apoptosis might be the main mechanism in cell deaths triggered by CSE and pyroptosis might have the minor role.

p21 and p27 have been known to induce cell-cycle arrest. However, their activities extended to the regulation of apoptosis beyond cell-cycle regulation(29). p21 could protect cells from p-53 mediated apoptosis and could interact with pro-caspase 3 to inhibit caspase 3 activation(28, 35). The function of p27 in apoptotic process is still uncertain. In tumor cell lines, it is suspected to act as pro-apoptotic function. However, p27 in normal cell lines has been shown to prevent apoptosis as p27 products prevented pro-caspase 3 activation(36). When BEAS-2B cells were treated with CSE, p21 and p27 overexpression were observed. This could explain the reason why cell survival rather than cell deaths was demonstrated under 2.5-5.0% CSE stimulation.

The main strength of this study is to attest the pryoptosis of bronchial epithelial cells induced by cigarette smoke. As much as we know, this is the first study to prove the CSE-related pyroptosis. However, there are also some

limitations in this study. First, signaling pathway of pyroptosis could not be evaluated. Although many studies demonstrated caspase 1-dependent cell death, specific molecular marker for pyroptosis and precise signaling pathway still remain unknown. How caspase 1 activation lead to the pore formation on cell wall should be investigated. Second, the measurement of secreted cytokines from pyroptotic cells was not performed. Compared with apoptosis, pyroptosis is pro-inflammatory cell death(32). Third, necrosis of BEAS-2B cells by CSE was not considered. In fact, necrosis did not indicate specific pathway of cell death(11). However, the features of necrosis could be distinguished from that of apoptosis under cigarette smoke exposure(37). Finally, CSEs used in each experiment were not same because of repetitive experiments. Although we tried to make CSE under the same circumstance, the ingredient of each CSE might differ from the others and this difference could affect the outcome.

In conclusion, cigarette smoke could induce caspase 1-dependent cell death called pyroptosis of bronchial epithelial cells. Moreover, other cell death mechanisms as well as pyroptosis contributed to the cell death by cigarette smoke.

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국문 초록

서론: 약 4,000 여 종류의 화합물로 구성된 흡연 추출물은 다양한 방법으로 세포 사멸을 유도할 수 있다. 현재까지 흡연 추출물이 세포 자살 (apoptosis)과 괴사 (necrosis)를 일으킬 수 있다고 알려져 있지만, caspase 1 에 의해 유발되는 pyroptosis 는 현재까지 밝혀진 바가 없다. 이에 본 연구에서는 흡연 추출물에 의한 기관지 상피세포의 pyroptosis 를 밝히고자 한다.

방법 및 결과: BEAS-2B 세포가 각기 다른 농도의 흡연 추출물로 처리되었다. 흡연 추출물을 12 시간, 24 시간, 그리고 48 시간동안 처리한 후에 MTT 정량법을 시행하여 세포의 생존 정도를 측정하였다. 24 시간 처리하였을 때 흡연 추출물의 농도가 7.5% 이상이면 세포 생존이 유의하게 감소하였다. 그러나 2.5% 농도의 흡연 추출물 하에서는 오히려 세포 생존이 증가하였다.

Pyroptosis 는 caspase 1 활성화에 의해 유도되기 때문에 caspase 1 의 활성도가 측정되었다. 흡연 추출물을 처리하였을 때, caspase 1 의 발현 증가가 western blot 분석으로 확인되었다. 또한 caspase 1 활성도 측정 키트로 측정된 caspase 1 의 활성도 역시 흡연 추출물에 의해 증가되었다. Pyroptosis 의 주된 특징이 세포 벽 공동 (pore) 형성이기

때문에, EtBr 흡수 정도가 측정되었다. 또한 공동 형성에 따른 세포 사멸이 MTT 정량법 및 LDH 분비 정량법으로 측정되었다. 흡연 추출물을 처리하였을 때 세포의 EtBr 흡수와 세포 사멸이 증가하였다. 그러나 caspase 1 억제제 (Z- YVAD-FMK 10μM)가 함께 처리되었을 때 EtBr 흡수와 세포 사멸은 유의하게 감소하였다. 유세포분석을 시행하였을 때 흡연 추출물이 처리된 세포는 세포의 크기가 증가되었는데, 이는 흡연 추출물에 의한 동공 형성 및 이에 따른 세포 부종을 시사한다.

그렇지만 세포 사멸 전체가 pyroptosis 만으로 설명될 수 없기 때문에, 세포 자살(apoptosis)과 자가소화작용 (autophagy)에 대한 특징적인 지표들 역시 같이 측정되었다. 흡연 추출물은 caspase 3 를 활성화 시켰고 이는 PARP 단백의 분해를 매개하였다. 또한, Bcl-xL 의 발현 수준이 흡연 추출물에 의해 감소되었다. Annexin V 유세포분석 역시 흡연 추출물에 의해 세포 자살이 증가함을 보여주었다. 마지막으로, caspase 3 억제제 (Z-DEVD-FMK 10μM) 가 흡연 추출물과 함께 처리되었을 때 LDH 분비 정량 및 MTT 정량 분석에서 세포 사멸이 감소하는 것이 확인되었다.

자가소화작용의 지표인 Beclin 1 과 LC-3B 역시 흡연 추출물에 의해 활성화되었다. 세포 자살 및 자가소화작용의 지표 외에도 세포 노화의 지표인 p21 과 p27 의 발현 역시 평가하였다. 흡연 추출물 처리 시에 p21 및 p27 의 발현 또한 증가하였는데, 이는 특정 농도

의 흡연 추출물 처리 하에서는 세포 사멸이 감소하고 오히려 세포 생존이 증가하는 결과를 설명해준다.

결론: 결론적으로 흡연 추출물은 caspase 1 활성화를 통한 기관지 상피세포의 pyroptosis 를 유도한다. 또한, pyroptosis 외에도 세포 자살이나 자가소화작용과 같은 다른 세포 사멸 기전 역시 흡연 추출물에 의한 세포 사멸에 영향을 준다.

주요어 : Pyroptosis, caspase 1, 흡연 추출물, 기관지 상피세포

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