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ORGAN TOXICITY AND MECHANISMS

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Immunohistochemical Detection of Apoptotic Proteins, p53/Bax and JNK/FasL Cascade, in the Lung of Rats Exposed to Cigarette Smoke

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Abstract Lung disease is the leading and second-leading cause of death in women and men in Taiwan, respectively. Epidemiological studies conducted in Taiwan have shown that cigarette smoking is the principal risk factor of lung disease, but little is known about the association between apoptosis and cigarette smoke (CS)-induced lung pathogenesis. We designed an animal exposure system to study signal proteins involved in the process of apoptosis induced by smoking in rat terminal bronchiole. Rats were exposed to CS in doses of 5, 10, and 15 cigarettes, respectively, and the exposure lasted for 30 min, twice a day, 6 days a week for 1 month. Following which the rats were sacrificed and the lung tissues were analyzed by histopathological methods. The terminal bronchioles revealed mild to severe inflammation according to the doses of CS and marked lipid peroxidation, lymphocyte infiltration, congestion, and epithelial emphysema of alveolar spaces were also noted. Using an in situ cell death detection kit (TA300), the association of CS with apoptosis was determined in a concentration-dependent manner. Immunohistochemical evaluation showed that CS treatment produced an increase in the cellular levels of Bax, t-Bid, cleaved caspase-3, phospho-p53, phospho-JNK, and FasL but a decline in Bcl-2 and Mcl-1 ($p < 0.001$ for all) in rat terminal bronchioles. The results provided evidences suggesting that exposure to CS not only induced apoptosis, but also involved p53/Bax and JNK/FasL cascade pathway.

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Abbreviations CS: Cigarette smoke · Bax: Bcl-2 associated X protein · t-Bid: Truncated Bid · Bcl-2: B cell lymphoma-2 · MAPK: Mitogen-activated protein kinases family · JNK: c-Jun N-terminal kinase · Fas-L: Fas ligand · DAB: Diaminobenzidine · iNOS: Inducible NO synthase · NOx: Nitrogen oxides · HPF: High-power-field

Introduction

Cigarette smoke (CS) is a major risk factor which causes the development of many cancers, including cancers of the larynx, oral cavity, pharynx, esophagus, pancreas, kidney, bladder, and lung (Czekaj et al. 2002; Shishodia and Aggarwal 2004). Smokers have a high incidence of lung cancer, which is the most common cause of cancer in Western countries, accounting for more deaths than those caused by prostate, breast, and colorectal cancers combined (Greenlee et al. 2001; Villeneuve and Morrison 1995). Recent estimates indicate that CS causes approximately 80–90% of lung cancer in Taiwan (Ko et al. 1997). However, the mechanisms by which CS induces lung cancer remain unclear, and among the 3,800 compounds identified in CS, a large number of them exert mutagenic and carcinogenic activity (Vineis and Caporaso 1995).

Recent studies have found that exposure to CS results in oxidative stress stimulation of inducible NO synthase (iNOS) and c-fos via regulation of protein tyrosine phosphorylation and MAP kinase, which in turn may promote lung pathogenesis (Chang et al. 2001). CS has been reported to have the ability to induce DNA single strand breaks in cultured human and rodent cells (Fielding et al. 1989; Leanderson and Tagesson 1992; Nakayama et al. 1984; Spencer et al. 1995; Stone et al.

1994, 1995). It was previously reported that a positive correlation exists between the number of cigarettes smoked per day and both the 8-hydroxyguanosine (8-OH-G) levels and their repair activities in human peripheral leukocytes and lung tissue (Asami et al. 1996, 1997). Peroxynitrite was identified in aqueous CS fraction (Muller et al. 1997), and this has been shown to react with guanine and DNA in vitro to form 8-nitroguanine (8-NO_2-G) (Yermilov et al. 1995a, b). Moreover, it has recently been found that gaseous NOx can induce 8-NO_2-G formation in human lung fibroblast cells and in tobacco cigarette smokers (Hsieh et al. 2001) and smoke-exposed Wistar rats (Hsieh et al. 2002). Although, detailed mechanisms of CS have been established in oxidative stress, DNA damage and aberrant proliferation, CS-regulated apoptosis remains unclear in vivo (De Flora et al. 2003).

Inappropriate regulation of apoptosis is associated with a variety of diseases, ranging from neurodegenerative disorders to malignancy (Thompson 1995). Past studies suggest that apoptosis might influence the malignant phenotype and promote tumor progression (Lowe and Lin 2000). Apoptosis involves two main pathways: death receptor and mitochondria death pathway (Herr and Debatin 2001). The apoptotic death receptor pathway is induced by members of the death receptor superfamily such as Fas (CD95). The Fas ligand (FasL) bonds to its receptor, forming a death-inducing complex with the adaptor molecule Fas-associated death domain (FADD) and finally caspase cascade activation is required for apoptosis. The mitochondrial death pathway is controlled by members of the Bcl-2 family, including the anti-apoptotic Bcl-2 and Bcl-XL proteins and the proapoptotic Bax and Bid proteins. Death signals stimulated cytochrome c, Apaf-1, and other possible factors released from the mitochondria. The two-apoptotic death pathways converge at activation of caspase-3 (Luo et al. 1998; Li et al. 1998).

In addition to caspases, the members of the mitogen-activated protein kinases family (MAPK) are also pathways that mediate apoptosis. The MAPK superfamily of serine/threonine kinases is activated by numerous extracellular stimuli and is involved in signal transduction cascades that play an important regulatory role in cell growth, differentiation, and apoptosis (Cobb and Goldsmith 1995; Chan-Hui and Weaver 1998). Three major mammalian MAPKs have been described: the extracellular signal regulating kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. However, the diverse MAPK members are activated in response to different extracellular stimuli and have distinct downstream targets, thus serving different roles in cellular responses. It is worth mentioning that JNK is activated by UV irradiation, DNA damage, hydrogen peroxide, heat, and osmotic shock, resulting in apoptotic cell death (Ichijo 1999).

In the past two decades, significant progress has been made in understanding the molecular and cellular pathogenesis of lung cancer. However, the mechanism behind the CS-induced apoptosis and related proteins remains unclear. To elucidate the association between apoptosis and CS-induced lung pathogenesis, an animal exposure system was designed to investigate apoptosis and to evaluate the expression of related proteins using immunohistochemical detection method in the terminal bronchiole areas of the lung tissue of rats exposed to CS.

Materials and methods

Chemicals

Tris citrate buffer, xylene, hematoxylin, H_2O_2, and diaminobenzidine (DAB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). In-situ apoptosis detection kit, TACS Blue Labe and Kit (TA300) was purchased from R and D Systems Inc. (MN, USA). Polyclonal antibody against phospho-p53 (Ser392) was obtained from Transduction Lab. (KY, USA). Antibodies against FasL, Bcl-2, Mcl-1, Bax, t-Bid, and cleaved caspase-3 were from Santa Cruz Biotechnology (CA, USA), and phospho-JNK (Thr183/Tyr185) was from Cell Signaling Tech. (MA, USA).

Animal treatment

Male Wistar rats (120 ± 10 g body weight at 6-week old) were purchased from the National Science Council Animal Center, Taiwan. These animals were housed six per cage in an environmentally controlled animal room, and water was provided ad libitum. All animals were handled according to the guidelines of the Taiwan Society for Laboratory Animals Sciences for the care and use of laboratory animals. A total of 24 rats were divided into four exposure groups. For the exposure test, rats were placed in whole-body exposure chambers and exposed to smoke from 5, 10, or 15 cigarettes (New Paradise, Taiwan) simultaneously or filtered room air as control. The filtered breathing air was introduced into the chamber at a flow rate of 200 l/min. The exposure was carried out twice a day, 6 days per week for over 1 month, with each exposure lasting for 30 min. During the exposures, the temperature was maintained at 22–25°C, and the relative humidity was approximately 40%. One month after cessation of exposure, the rats were sacrificed by decapitation, and the lungs were immediately excised for immunohistochemistry determination of apoptosis-related proteins.

Autopsy and histology

Immediately after death, a complete necropsy was performed and the lung was examined. The lung was washed with physiologic saline and inspected for gross lesions. Tissues were fixed in 10% buffered formalin,

processed for histological examination according to the lung tissue and stained with hematoxylin and eosin. The morphology, number, and location of any lesion observed were registered.

In-situ apoptosis detection

After excision, the lung tissues were fixed in 1,040 buffered-formalin solution for 18–24 h, dehydrated, embedded in paraffin, and cut into sections of 5 μm thickness. Prior to the labeling reaction, the samples were deparaffinized by xylenes and ethanol. The labeling reaction mixtures were prepared just before use according to the method of apoptosis detection kits (R and O Systems Inc., MN, USA). Small biopsy samples were covered with 50 μl labeling reaction mixtures and incubated at 37°C for 1 h. Samples were immersed in stop buffer for 5 min at 18–24°C and washed twice with dH_2O. The samples were then mixed using 50 μl detection buffer and incubated at 18–24°C for 60 min. After washing, the samples were incubated with 50 μl of TACS Blue Label (TBL) for 2–5 min. Samples stained with TBL were counterstained with Nuclear Fast Red. Cells containing fragmented nuclear chromatin characteristic of apoptosis exhibit a blue nuclear staining that may become very dark after labeling, and this intense blue staining is typically associated with cell condensation. The preparation of solution and buffer was performed following the description for the commercial kit (see the procedure of Apoptosis Detection Kit TA300, R and D Systems Inc. for details). Six randomly selected fields (0.2 mm^2) from six lung tissues of every group at a final magnification of 400 folds were counted in each section and the results were expressed as the total TBL-positive cells per mm^2.

Immunohistochemical analysis

After being excised from the animals, the lungs were fixed in 10% buffer-formalin solution for 18–24 h, dehydrated, embedded in paraffin and cut into sections of 5 μm thickness. To perform the immunohistochemistry, the sections were deparaffinized in xylene, rehydrated in 0.05 M Tris buffer, pH 7.6, for 10 min and boiled in 0.01 M citrate buffer, pH 6.0, for 5 min. The sections were then removed and allowed to cool at room temperature for 20 min and rinsed two times with TBS for a total of 30 min. Endogenous peroxidase activity was blocked by 15 min incubation in 3% hydrogen peroxide. To increase antigenic exposure, tissue sections were incubated in 0.1% Triton X-100 for 45 min at room temperature.

Following this, the samples were incubated with diluted primary antibodies, including anti-FasL, Bcl-2, t-Bid, Mcl-1, phospho-p53, phospho-JNK, Bax, cleaved caspase-3 polyclonal antibodies, for 45 min at room temperature. After rinsing two times with TBS for a total of 20 min the bound primary antibodies were detected by sequential incubation with biotinylated secondary antibody (Biotinylated anti-rabbit or anti-mouse immunoglobins, LSAB kit from DAKO) for 30 min, streptavidin peroxidase (LSAB kit from DAKO) for 15 min, and DAB for 5–10 min, at room temperature with two rinses of TBS in between. The sections were then washed with distilled water and counterstained with Mayer's hematoxylin. After dehydration and mounting, the expressions of these detection proteins in rat lung tissue were assessed by microscopic examination of the immunoperoxidase staining. The negative control for each experiment was a DAKO antibody diluent with background reductional compound, which is intended for use as a diluent in the preparation of primary antibodies and negative control reagents. The positively staining area, brown in color, was determined by Leica Q500 MC Image processing and analysis system against the negatively staining region shown relatively as blue in color.

Statistical analysis

Data reported are means ± standard deviation of six different animals and evaluated by one-way analysis of variance (ANOVA). Significant differences were established at $p < 0.05$.

Results

Lung injury caused by CS

To study the role of CS in lung pathogenesis in vivo, rats were exposed to various concentrations of CS. The rats were exposed to gas-phase CS in a chamber twice a day, 30 min for each time and 6 days per week for over 1 month. The experimental conditions were designed to mimic passive smoke exposure of humans in a confined room. One month after cessation of exposure, injury induced by CS included lung inflammation, emphysema dilatation and peribronchiole fibrosis in the terminal bronchioles and alveolar duct area. The injury caused by CS was directly related to the number of cigarettes (data not shown).

In-situ apoptosis induced by CS

Following 1 month of CS treatment, the rat lung tissue showed typical apoptotic features: membrane blebbing, chromatin condensation, nuclear DNA fragmentation, and apoptotic bodies. A histogram of TBL-positive cells per mm^2 with representative photographs, as shown in Fig. 1, reveals that exposure of rats to the smoke from 5 to 15 cigarettes produced a concentration-dependent increase in the TBL-positive cells. Table 1 showed a

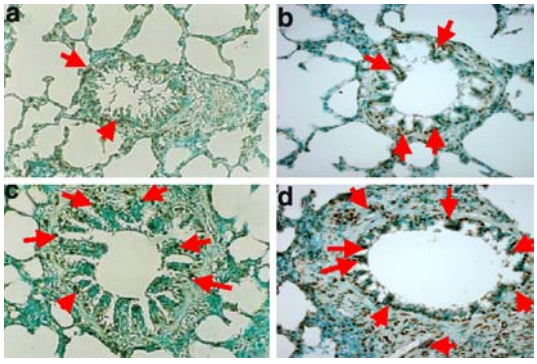

Fig. 1 Immunohistochemical examination of the expression of apoptosis in rat exposed smoking lung tissue .The rats were exposed to the smoke from (**a**) 0; (**b**) 5; (**c**) 10; or (**d**) 15 cigarettes for 0.5 h per day, twice a day, 6 days a week for 1 month. The *arrow* indicates myoepithelial cells undergoing apoptosis. The level of positive stain was quantified and shown in Table 1. Mean ± SD of six different animals. $^{*}p < 0.001$ compared with the control

Table 1 Qualtitation of apoptosis expression on cigarette-induced rat lung lesion tissues

Treatment cigarette No.	Mean ± SD[a] (mm^2)	Fold of control
0	0.34 ± 0.03	–
5	$0.43 \pm 0.03^{*}$	1.26
10	$0.50 \pm 0.03^{*}$	1.47
15	$0.63 \pm 0.03^{*}$	1.85

[a]Mean ± SD of six different animals
*$p < 0.001$, compared with control group

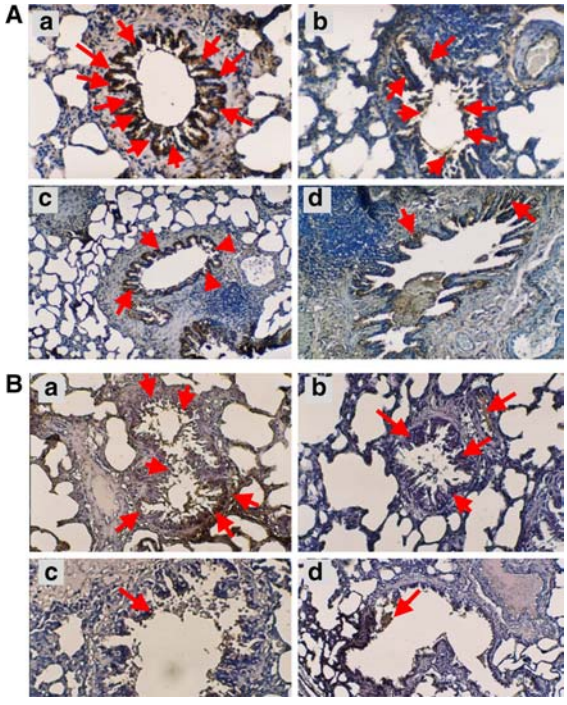

Fig. 2 Immunohistochemical evaluation of the effect of gas-phase CS on the expression of (**A**) Bcl-2 and (**B**) Mcl-1. Lung sections obtained from the rats exposed to the smoke from (**a**) 0; (**b**) 5; (**c**) 10; or (**d**) 15 cigarettes for 0.5 h per day, twice a day, 6 days a week for 1 month. The expressions of Bcl-2 and Mcl-1 were detected by anti-Bcl-2 and Mcl-1 antibodies and biotin-avidin peroxidase system. *Arrows* indicated positive stain areas apparent in the terminal bronchioles of lung. The level of positive stain was quantified and shown in Table 2. Mean ± SD of six different animals. $^{*}p < 0.001$ compared with the control

significant increase of about 1.26-, 1.47-, and 1.85-folds ($p < 0.001$ for all). These results indicated that expression of apoptosis was significantly increased in rat lungs exposed to the smoke from 5 to 15 cigarettes.

Effect of CS on bcl-2 family

Investigations of the bcl-2 gene family have shown a complex network regulating apoptosis. The Bcl-2 protein family is an integral membrane protein that regulates apoptosis in several numerous biological systems (Danial and Korsmeyer 2004). We examined the cellular level of Bcl-2, Mcl-1, Bax, and t-Bid in lung tissues from CS-exposed rats. The expression of Bcl-2 and Mcl-1 (anti-apoptotic proteins) were significantly decreased in rat lungs exposed to the smoke from 5 to 15 cigarettes (Fig. 2) with maximal reduction folds of control about 0.75- and 0.49-folds, respectively (Table 2); in addition, the expressions of Bax and t-Bid (proapoptotic proteins) were elevated after exposure to CS (Fig. 3A, B) and reached the maximum levels at about 3.29- and 1.75-folds in rat lungs exposed to the smoke from 15 cigarettes ($p < 0.001$ for all, Table 3).

Effect of CS on caspase cleavage

Caspases play a pivotal role during apoptosis after treatment with an inducing agent. Immunohistochemical analysis was used to understand whether caspases were activated by CS in rat lungs. We next examined the expression of cleaved caspase-3. Caspases-3 are cytosolic proteins that exist normally as inactive precursors with higher molecular weight. It is cleaved proteolytically into low molecular weight (20 kDa) when cell undergoes apoptosis. Data show that the cleavage caspase-3 was apparently increased after exposure to CS (Fig. 4 and Table 4). The result suggested that CS induced the activation of caspase-3 in rat lungs.

Effect of CS on p53 phosphorylation

The overexpression of Bax accelerates apoptotic death induced by different stress in many cell lines. It has been reported that in cells with stably expressed p53 a continuous increase in Bax expression might be responsible for the apoptosis (Oltvai et al. 1993). Our findings showed that rat lungs expressed relatively high level of Bax (Fig. 3A) and an increase in phosphorylation of p53

Table 2 Levels of Bcl-2 and Mcl-1 expression on cigarette-induced rat exposed smoking lung tissue

Treatment cigarette No.	Bcl-2		Mcl-1	
	Mean ± SD[a] (mm^2)	Fold of control	Mean ± SD[a] (mm^2)	Fold of control
0	0.72 ± 0.03	–	0.66 ± 0.04	–
5	$0.56 \pm 0.03^*$	0.78	$0.47 \pm 0.04^*$	0.71
10	$0.36 \pm 0.03^*$	0.50	$0.41 \pm 0.02^*$	0.62
15	$0.18 \pm 0.02^*$	0.25	$0.34 \pm 0.03^*$	0.51

[a]Mean ± SD of six different animals
*$p < 0.001$, compared with control group

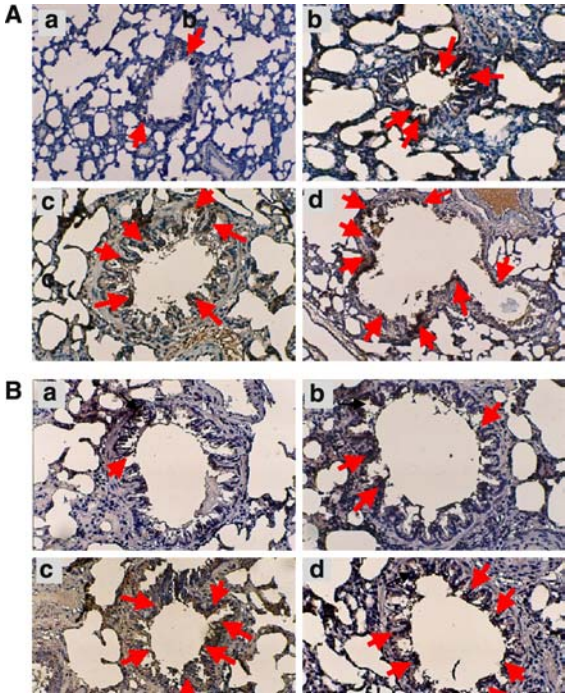

Fig. 3 Immunohistochemical evaluation of the effect of gas-phase CS on the expression of (**A**) Bax and (**B**) t-Bid. Lung sections obtained from the rats exposed to the smoke from (**a**) 0; (**b**) 5; (**c**) 10; or (**d**) 15 cigarettes for 0.5 h per day, twice a day, 6 days a week for 1 month. The expressions of Bax and t-Bid were detected by anti-Bax and t-Bid antibodies and biotin-avidin peroxidase system. *Arrows* indicated positive stain areas apparent in the terminal bronchioles of lung. The level of positive stain was quantified and shown in Table 3. Mean ± SD of six different animals. *$p < 0.001$ compared with the control

(Fig. 5) showed a significant and gradual increase of about 1.50-, 2.00-, and 2.43-folds ($p < 0.001$ for all, Table 5) following CS treatment that, thereby, facilitated the induction of apoptosis.

Effect of CS on JNK/ FasL signaling

Previous studies have also shown that an increase in the phosphorylation of JNK was observed in the cigarette-treated endothelial cells (Hoshino et al. 2005; Raveendran et al. 2005), and the persistence of this activation led to the activation of FasL, an AP-1 target gene. Release of FasL initiated apoptosis through Fas death receptor, which, subsequently, truncated Bid, implying an association between the cigarette-induced apoptosis and the activated JNK/FasL signaling (Lei et al. 1998; Raveendran et al. 2005). Whether CS-induced apoptosis is also modulated by JNK/FasL was examined. In rat lung tissues, the phosphorylation of JNK was significantly increased after exposure to CS ($p < 0.001$). The expression of phospho-JNK showed dose-dependence in CS-exposed rats (Fig. 6A). Similar results were also observed in the study of FasL (Fig. 6B). There was a 1.78–2.89 folds expression of phospho-JNK and 1.53–2.47 folds expression of FasL (Table 6) in the CS-exposed rats compared with that of the control, respectively.

Discussion

Cigarette smoking is known to be a risk factor in cancer development. However, the underlying mechanisms, in particular the contribution of gas-phase smoke constituents, have not yet been completely identified. The experimental conditions were designed to mimic passive smoke exposure of humans in a confined room. The rats were exposed to gas-phase CS in a chamber 30 min twice a day and 6 days per week for over 1 month. These rat lung lesions showed blue positive staining indicating the level of apoptosis in a dose-dependent manner by in-situ apoptosis detection (Fig. 1). Apoptosis was reported to contribute to the high rate of cell loss in malignant tu-

Table 3 Level of Bax and t-Bid expression on cigarette-induced rat exposed smoking lung tissue

Treatment cigarette No.	Bax		t-Bid	
	Mean ± SD[a] (mm^2)	Fold of control	Mean ± SD[a] (mm^2)	Fold of control
0	0.24 ± 0.04	–	0.24 ± 0.03	–
5	$0.43 \pm 0.05^*$	1.79	$0.34 \pm 0.02^*$	1.42
10	$0.51 \pm 0.03^*$	2.13	$0.39 \pm 0.04^*$	1.63
15	$0.79 \pm 0.05^*$	3.29	$0.42 \pm 0.02^*$	1.75

[a]Mean ± SD of six different animals
*$p < 0.001$, compared with control group

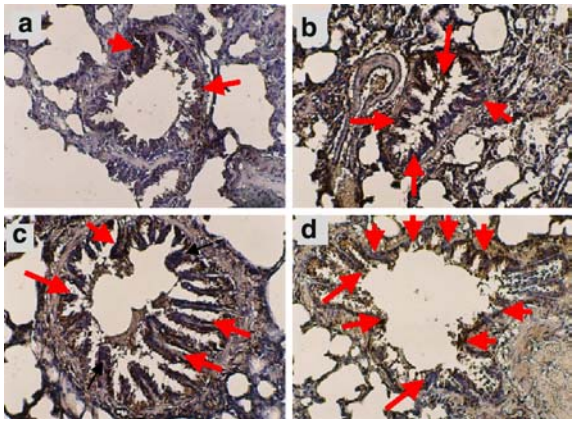

Fig. 4 Immunohistochemical evaluation of the effect of gas-phase CS on the expression of cleaved caspase-3. Lung sections obtained from the rats exposed to the smoke from (**a**) 0; (**b**) 5; (**c**) 10; or (**d**) 15 cigarettes for 0.5 h per day, twice a day, 6 days a week for 1 month. The expression of cleaved caspase-3 was detected by anti-cleaved caspase-3 antibody and biotin-avidin peroxidase system. *Arrows* indicated positive stain areas apparent in the terminal bronchioles of lung. The level of positive stain was quantified and shown in Table 4. Mean ± SD of six different animals. $^{*}p < 0.001$ compared with the control

Table 4 Level of cleaved caspase-3 expression on cigarette-induced rat exposed smoking lung tissue

Treatment cigarette.No.	Mean ± SD[a] (mm^2)	Fold of control
0	0.18 ± 0.02	–
5	0.25 ± 0.03*	1.39
10	0.40 ± 0.03*	2.22
15	0.49 ± 0.04*	2.72

[a]Mean ± SD of six different animals
*$p < 0.001$, compared with control group

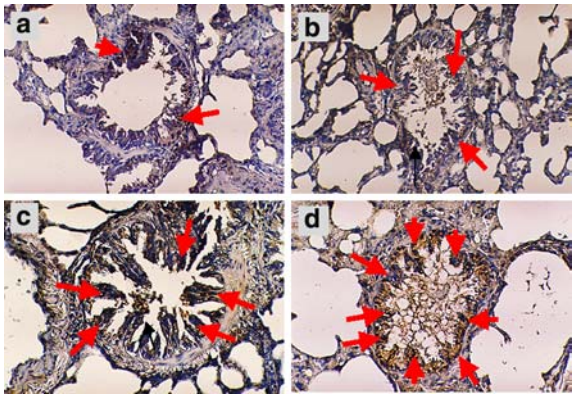

Fig. 5 Immunohistochemical evaluation of the effect of gas-phase CS on the expression of phospho-p53. Lung sections obtained from the rats exposed to the smoke from (**a**) 0; (**b**) 5; (**c**) 10; or (**d**) 15 cigarettes for 0.5 h per day, twice a day, 6 days a week for 1 month. The expression of phospho-p53 was detected by anti-phospho-p53 antibody and biotin-avidin peroxidase system. *Arrows* indicated positive stain areas apparent in the terminal bronchioles of lung. The level of positive stain was quantified and shown in Table 5. Mean ± SD of six different animals. $^{*}p < 0.001$ compared with the control

Table 5 Level of p-p53 expression on cigarette-induced rat exposed smoking lung tissue

Treatment cigarette No.	Mean ± S.D[a] (mm^2)	Fold of control
0	0.28 ± 0.04	–
5	0.42 ± 0.03*	1.50
10	0.56 ± 0.04*	2.00
15	0.68 ± 0.04*	2.43

[a]Mean ± SD of six different animals
*$p < 0.001$, compared with control group

mors and, moreover, promote tumor progression in previous studies (Lowe and Lin 2000; Thompson 1995).

What triggers apoptosis during lung tumor development? A variety of signals appear to be important. Increasing evidence suggests that free radicals are involved in many of the chronic diseases associated with smoking. Molecular toxicological investigations demonstrated that CS can produce DNA single strand breaks (Fielding et al. 1989; Leanderson and Tagesson 1992; Nakayama et al. 1984; Spencer et al. 1995; Stone et al. 1994, 1995), elevated expression of heme oxygenase (Muller and Gebel 1994) and altered regulation of the

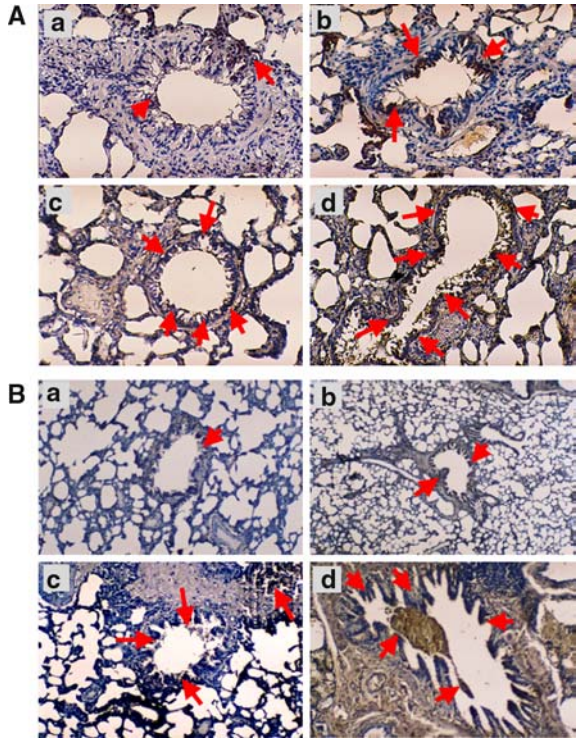

Fig. 6 Immunohistochemical evaluation of the effect of gas-phase CS on the expression of (**A**) phospho-JNK and (**B**) FasL. Lung sections obtained from the rats exposed to the smoke from (**a**) 0; (**b**) 5; (**c**) 10; or (**d**) 15 cigarettes for 0.5 h per day, 6 days a week, twice a day, for 1 month. The expression of phospho-JNK was detected by anti-phospho-JNK and anti-FasL antibodies and biotin-avidin peroxidase system. *Arrows* indicated positive stain areas apparent in the terminal bronchioles of lung. The level of positive stain was quantified and shown in Table 6. Mean ± SD of six different animals. $^{*}p < 0.001$ compared with the control

Table 6 Level of p-JNK and FasL expression on cigarette-induced rat exposed smoking lung tissue

Treatment cigarette No.	*p*-JNK		FasL	
	Mean ± SD[a] (mm^2)	Fold of control	Mean ± SD[a] (mm^2)	Fold of control
0	0.18 ± 0.05	–	0.30 ± 0.03	–
5	0.32 ± 0.03*	1.78	0.46 ± 0.03*	1.53
10	0.43 ± 0.03*	2.39	0.63 ± 0.03*	2.10
15	0.52 ± 0.04*	2.89	0.74 ± 0.03*	2.47

[a]Mean ± SD of six different animals
*$p < 0.001$, compared with control group

c-fos gene at the transcriptional level (Muller 1995). Data also showed that exposure to CS results in oxidant stress leading to the stimulation of iNOS and c-fos via regulation of protein tyrosine phosphorylation and MAP kinase, which in turn may promote lung pathogenesis (Chang et al. 2001).

During the process of apoptosis induced by stress from sources such as ROS, anticancer drugs, UV irradiation, Fas (Seimiya et al. 1997; Butterfield et al. 1997; Juo et al. 1997), were associated with or required for apoptosis. The goal of the following set of experiments was to determine which pathway is associated with the CS-induced apoptosis in rat lungs. Apoptosis-related molecules may have an opportunity to be the candidate. The Bcl-2 family of proteins constitutes a critical intracellular checkpoint in the intrinsic pathway of apoptosis, as the mitochondria pathway. Mammals possess an entire family of Bcl-2 proteins including proapoptotic as well as antiapoptotic members. Importantly, it has been demonstrated that the gene products of Bcl-2 and Bax play important roles in apoptotic cell death (Oltvai et al. 1993; Jacobson and Raff 1995). In the Bcl-2 family, the ratio of Bcl-2 and Bax proteins has been recognized as a key factor in regulating the apoptotic process (Adams and Cory 1998; Green and Reed 1998). In the present study, the increase in CS-induced apoptosis was associated with an increase in levels of Bax protein (Fig. 3A), which heterodimerizes with and thereby inhibits Bcl-2 (Fig. 2A). Our study has demonstrated that CS may alter the expression of Bcl-2 and Bax and, therefore, lead to the apoptosis of rat lung tissues.

Because CS increased the amount of Bax, which is one of the p53 target genes, we next examined whether the CS-induced apoptosis may be mediated by the activation of p53. The phosphorylation level of p53 showed a significant increase of about 1.50-, 2.00-, and 2.43-folds ($p < 0.001$ for all) following exposure of 5–15 cigarettes (Fig. 5). These results indicated that the CS-induced apoptosis was associated with an induction of p53 phosphorylation that subsequently modulated the expression of Bcl-2 family.

Of the death receptor pathway processes, FasL-mediated apoptosis followed a death-inducing signal complex (DISC) formation showing that the members presented have a death receptor Fas, FADD and caspase-8. The activated form of caspase-8 may activate in turn either caspase-3 or cleave Bid. Activated Bid triggers apoptosis by mitochondrial translocation, which stimulates cytochrome c release (Yin 2000). In Fas/TNF system, caspase-3 activation is mediated by caspase-8 or caspase-10 (Alnemri 1997). Caspase-3 has been shown to play a central role in the process of apoptosis induced by many stimuli (Tewari et al. 1995), as an effector caspase. Moreover, it has been demonstrated that cleavage of Bid mediates caspase-8-induced cytochrome c release from the mitochondria (Luo et al. 1998). It strongly suggested that Bid cleavage is necessary for CS-related caspase-3 activation and apoptosis release (Fig. 4). Some studies reported that caspases can modulate JNK activation in Fas- or TNFα-induced apoptosis (Juo et al. 1997; Roulston et al. 1998), indicating that the stress-activated kinases perhaps induced caspase activation. Thus, we observed that CS induced an increase in JNK activity related to FasL (Fig. 6 and Table 6).

The data obtained in the present study led to the following conclusions regarding the regulation of rat lung tissues destroyed by CS: (1) CS can induce apoptosis of rat lung tissues in a concentration-dependent manner, (2) CS-induced caspase activation leads to death of rat lung tissues, (3) CS induces apoptosis through a mechanism involving the release of apoptosis-related proteins from the mitochondria, and (4) CS-induced death in rat lung tissues requires JNK/FasL to activate an upstream of caspases.

In summary, this study is consistent with other studies demonstrating that CS induces lung injury including apoptosis via the two mechanisms involved, that is, (1) phosphorylation of JNK MAPK pathway followed by either activation of FasL or Bcl-2, and (2) an increase in Bax with the expression of phospho-p53 stimulated, thus leading to the activation of cleaved caspase-3. This effect may induce apoptosis and may be an important pathway in the lung pathogenesis of CS. These findings may help develop a novel strategy for prevention of lung cancer by understanding the signal cascade of CS-induced lung pathogenesis.

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