Free Radical Research, October 2011; 45(10):1232–1240

informa
healthcare

Lysosomal membrane permeabilization contributes to elemene emulsion-induced apoptosis in A549 cells

LONG-JIE LI[1], LAI-FU ZHONG[2], LI-PING JIANG[3], CHENG-YAN GENG[3], TING-ZHUN ZHU[4], YING-HUI XU[4], QI WANG[5], YI QU[1], JING SHAO[2] & LI-JUAN ZOU[1]

[1]*Department of radiotherapy oncology, 2nd Affiliated Hospital of Dalian Medical University, 467 Zhong Shan Road, Dalian 116023, Liaoning, China,* [2]*Department of Toxicology, Dalian Medical University, No. 9, West Segment of South lushun Road, Lushunkou District, Dalian 116044, Liaoning, China,* [3]*China-Japanese Joint Institute for Medical and Pharmaceutical Science, Dalian Medical University, No. 9, West Segment of South lushun Road, Lushunkou District, Dalian 116044, Liaoning, China,* [4]*Department of Neurosurgery, 1st Affiliated Hospital of Dalian Medical University, 222 Zhong Shan Road, Dalian 116011, Liaoning, China,* [5]*Department of Respiratory Medicine, 2nd Affiliated Hospital of Dalian Medical University, Liaoning, China*

(Received date: 8 December 2010; Accepted date: 20 July 2011)

Abstract

Elemene is a broad-spectrum antitumor agent. In the present study, lysosomal membrane permeabilization (LMP) was detected after short elemene emulsion - exposure (12 h) that preceded a decrease of the mitochondrial membrane potential and DNA damage (24 h) in A549 cells. At later time points (36 h) elemene emulsion caused the appearance of A549 cells with apoptotic features, including apoptotic morphology, phosphatidylserine exposure, and caspase-3 activation. A significant increase in protein expression for cathepsin D was also observed utilizing Western blot analysis after exposure to elemene emulsion for 12 h. The present study showed that elemene emulsion induced the increased levels of reactive oxygen species (ROS) and depletion of glutathione (GSH) in A549 cells. Cells treated with pepstatin A, an inhibitor for cathepsin D, showed a significant inhibition in DNA damage, mitochondrial membrane permeabilization, caspase-3 activation, and phosphatidylserine exposure. These results demonstrated that apoptosis induced by elemene emulsion in A549 cells is mediated in part through LMP and lysosomal protease cathepsin D.

Keywords: *elemene; lysosome; apoptosis; oxidative stress*

Abbreviations: *SCLC, small cell lung cancer; NSCLC, non-SCLC; LMP, lysosomal membrane permeabilization; TNF, tumor necrosis factor; AO, acridine orange; ROS, reactive oxygen species; GSH, glutathione; DCFH-DA, 2,7-dichlorofluorescein diacetate; OPT, o-phthalaldehyde; S.D., standard deviation; LSD, least significant difference.*

Introduction

Lung cancer is the leading cause of cancer-related mortality and is responsible for over 1 million deaths each year worldwide [1]. The 2 main types of lung cancer are small cell lung cancer (SCLC) and non-SCLC (NSCLC); NSCLC accounts for approximately 85% of all cases of lung cancer [2]. Although chemotherapy is appropriate for many patients with NSCLC, there is a sense that the use of traditional chemotherapeutic agent has reached a therapeutic plateau [3] and 5-year survival from this disease is only 17% [4]. Obviously, developments of new medicine and new strategies to improve survival of patients with NSCLC are urgently needed. Recently, scientists have focused on the potential role of extracts of traditional Chinese medicinal herbs as alternative and complementary medications for NSCLC treatment [5].

Elemene, isolated from the traditional Chinese medical herb *Curcuma Wenyujin* [6], is a mixture of β-, γ-, and δ-elemene with β-elemene as the most abundant constituent. There is increasing evidence that

Correspondence: Prof. Li-Juan Zou, Department of radiotherapy oncology, 2nd Affiliated Hospital of Dalian Medical University, 467 Zhong Shan Road, Dalian 116023, Liaoning, China. Tel: +86 411 84671291. E-mail: lijuanzou1963@sina.com

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.3109/10715762.2011.607818

elemene possesses potent activities against malignant tumor cells. Elemene has been proved to inhibit the proliferation of human and rat glioblastoma cell [7,8]. The antitumor activity was also shown in leukemia cells [9–11]. In addition, preclinical studies have indicated that elemene is cytotoxic in several cancer cell lines, including ovarian cancer cells, laryngeal cancer cells and breast cancer cells [12–14]. Furthermore, previous results have suggested that elemene may be a potential therapy for NSCLC, because it induces apoptosis of NSCLC cells by activating the mitochondrial mediated pathway [15–17].

Two pathways can be involved in apoptosis induction: the extrinsic and intrinsic pathways. The extrinsic pathway is mediated by the interaction of death receptors with ligands, leading to activation of initiator caspase-8 and effector caspase-3. The intrinsic pathway is activated by many different stimuli and typically results in mitochondrial membrane depolarization, followed by activation of initiator caspase-9 and effector caspase-3 [18]. Apoptosis is most studied program that plays an essential role in the antitumor activity of elemene. Mitochondrial dysfunction and caspase activation are supposed to be involved in the apoptotic processes following exposure to elemene [15–17]. There is substantial evidence that lysosomal membrane permeabilization (LMP) not only has an early but, in fact, initiating role in apoptosis, and that mitochondrial dysfunction may be a consequence of earlier LMP [19–22]. LMP has been shown to occur in apoptosis under the influence of several stimuli, such as anti-cancer drugs, oxidative stress, tumor necrosis factor (TNF), and Fas [23–26].

The overall object of the present study is to further explore the cellular mechanisms of apoptosis induced by elemene emulsion in A549 cells. The time relationship of lysosomal and mitochondrial damage was determined to test the hypothesis that decrease in mitochondrial membrane potential and subsequent mitochondrial reactive oxygen species (ROS) are downstream events from LMP, and cathepsin D activation. The DNA damage and glutathione (GSH) depletion induced by elemene emulsion were also measured. Apoptosis was studied by examining cell morphology, phosphatidylserine exposure, and caspase-3 activation. Furthermore, we investigated whether pepstatin A, an inhibitor of cathepsin D, inhibited apoptosis induced by elemene emulsion.

Materials and methods

Chemicals, materials and mediums

Elemene emulsion was purchased from Jingang Pharmaceutical Co. (Dalian, China). 2,7-dichlorofluorescein diacetate (DCFH-DA), o-phthaladehyde (OPT), acridine orange (AO), rhodamine 123, Hoechst 33342, and pepstatin A were obtained from Sigma (St. Louis, MO). Normal melting points agarose and low melting point agarose were provided by Gibco BRL, Life Technologies (Paisley, UK). All tissue culture reagents, i.e., RPMI 1640 medium, fetal bovine serum, antibiotics (penicillin, streptomycin) and trypsin-EDTA were bought from Gibco BRL-Life Technologies (Grand Island, NY).

Cell culture and treatment

The human lung adenocarcinoma A549 cells were supplied by the American Type Culture Collection (ATCC) and were grown in RPMI 1640 medium containing 10% (v/v) fetal bovine serum and antibiotics [penicillin (100 IU/ml), and streptomycin (100 μg/ml)] at 37°C in 5% CO_2.

Cells were allowed to attach for 24 h before treatment with elemene emulsion. For the experiments, cultured cells were treated with various concentrations of elemene emulsion (final concentration: 5, 10, and 20 μg/ml) for 12 h, 24 h and 36 h. Control received only RPMI 1640 medium. For inhibition of the aspartic protease cathepsin D, cells were pretreated for 24 h with 50 μM pepstatin A and then concomitantly with elemene emulsion for 24 h or 36 h. Elemene emulsion were diluted in RPMI 1640 medium to working stocks immediately before use.

Assessment of apoptosis

Annexin V-Propidium Iodide staining

Normal, apoptotic, and necrotic cell were simultaneously distinguished using the Annexin V-Propidium Iodide (PI) kit according to the manufacturer's instruction (Kaiji, Nanjing, China). Briefly, control and treated cells were collected by trypsinization followed by staining with Annexin V and PI for 10 min. Single cell suspensions were analysed by flow cytometry.

Morphological assessment of apoptosis

After exposure to elemene emulsion, cells were rinsed with PBS. Nuclear morphology was assayed by fluorescence microscopy following Hoechst 33342 staining (final concentration 8 μg/ml for 15 min at 37°C). Imaging was carried out using an Olympus BX-51 fluorescent microscope with appropriate filter cubes.

Caspase-3 activity assay

After treatment with various concentrations of elemene emulsion, the caspase-3 activity of A549 cells was measured with the caspase-3 assay kit according to the manufacturer's instructions (Kaiji, Nanjing, China). Cells were assayed by use of the caspase-3 substrate Ac-DEVD-pNA. Briefly, 5×10^6 A549 cells were collected by trypsinization,

washed once with PBS, and lysed with lysis buffer. After incubation with Ac-DEVD-pNA at 37°C for 4 h, samples were read on a microplate reader at 405 nm (BIO-RAD 3550, USA).

Analysis of LMP

The AO is a metachromatic fluorophore which accumulates within acidic vacuolar compartments (lysosomes) due to protonation at low pH [27]. Damage of lysosomes was measured using the weak base AO, according to a protocol previously described [28]. Cells were incubated for 15 min with 5 μg/ml AO under otherwise ordinary culture conditions, then washed twice in PBS to remove the fluorescent dye from the media, followed by fluorescence spectrophotometer analysis (HITACHI, 650-60, Tokyo, Japan, excitation wavelength of 495 nm, emission wavelength of 530 nm).

Analysis of mitochondrial membrane potential

Changes in the mitochondrial membrane potential were measured using rhodamine 123 [28], which is a membrane-permeable fluorescent cationic dye. In brief, cells were incubated with rhodamine 123 (final concentration 1.5 μM) for 10 min at 37°C in a thermostatic bath with gentle shaking. The cells were centrifuged, and fluorescence from the supernatant was measured at an excitation wavelength of 490nm and an emission wavelength of 530 nm with a fluorescence spectrophotometer (HITACHI, 650-60, Tokyo, Japan).

Western blot analysis

Western blot analysis was performed using cytosolic fractions as previously described [19]. Equal amounts of cytosolic protein were separated by SDS-PAGE and transferred to nitrocellulose membrane. The blots were blocked with 5% nonfat milk in TBS with 0.1% Tween 20. Membranes were incubated with goat anti-cathepsin D polyclonal antibody overnight at 4°C. Finally membrane were incubated with HRP-conjugated secondary rabbit anti-goat antibodies at 37°C for 2 h, and assayed by an enhanced chemiluminescence plus (ECL+) detection system.

Comet assay

The Comet assay was performed as described by Singh and Stephens with slight modifications [29]. Cell suspension was mixed with 1% LMP agarose and added to fully frosted slides that had been covered with a layer of 1.5% NMP agarose. The cells were then lysed for 1 h at 4°C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, and 10 mM Tris, pH 10. After lysis, the slides were placed on an electrophoresis unit filled with fresh electrophoretic buffer (300 mM NaOH and 1 mM EDTA, pH 13) and left for 20 min for DNA unwinding and then electrophoresed for 30 min at 18 V and 200 mA. Afterwards, the slides were neutralized with 0.4 M Tris buffer (pH 7.5) and stained with 50 μl of ethidium bromide (20μg/ml). Finally, the slides were viewed using an Olympus BX-51 fluorescent microscope (excitation filter 549 nm, barrier filter 590 nm). Images of 50 randomly selected cells from each slide were analysed with Comet Assay Software Project casp-1.2.2 (University of Wroclaw, Poland). In the present study, tail moment was used as a parameter to assess DNA damage.

Intracellular ROS formation

The formation of intracellular ROS was measured using a fluorescent probe DCFH-DA as described previously [30]. The principle of the test is based upon the fluorescent probe DCFH-DA diffusing into the cells through cell membrane. Then, the DCFH-DA is hydrolyzed to non-fluorescent 2′,7′-dichlorofluorescin (DCFH). ROS cause oxidation of DCFH to a measurable fluorescent product, DCF. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly. H_2O_2 is the principle ROS responsible for the oxidation of DCFH-DA to DCF [31].

Following elemene emulsion treatment, cells were washed with cold PBS, suspended in PBS at 5×10^5 cells/ml, and incubated with DCFH-DA at a final concentration of 5 μM (40 min, 37°C in darkness). The relative fluorescence intensity was monitored using a fluorescence spectrophotometer (HITACHI, 650-60, Tokyo, Japan, excitation wavelength of 485 nm, emission wavelength of 530 nm).

Measurement of intracellular GSH

GSH was measured using a modified method of Hissin and Hilf [32]. The cells were exposed to elemene emulsion, washed twice with PBS, and then 5% trichloroacetic acid (0.4 ml) was added. After incubation at 4°C for 30 min to extract GSH, 50μl of the trichloroacetic acid extract was mixed with 0.8 ml 50 mM phosphate/5 μM EDTA buffer (pH 8) and the reaction was initiated by the addition of 50 μl OPT (1 mg/ml). The mixture was incubated at 37°C for 15 min in darkness. Fluorescence intensity was monitored by a fluorescence spectrophotometer (HITACHI 650-60, Tokyo, Japan, excitation wavelength of 350 nm, emission wavelength of 420 nm).

Statistical analysis

Statistical analysis was performed using SPSS v13.0 software. Data are expressed as mean ± standard

deviation (S.D.). The statistical significance of differences among groups was determined by analysis of variance with one-way ANOVA, followed by least significant difference (LSD) for multiple comparisons, as a post hoc test. The level of significance was set at $P < 0.05$ and $P < 0.01$ for all statistical analysis.

Results

Induction of apoptosis

Following exposure to elemene emulsion, cells were stained with Annexin V and PI and analysed by flow cytometry. The concentrations tested were 0, 5, 10, and 20 μg/ml and the points of time studied were 12 h, 24 h, and 36 h. There was no increase in apoptotic cells above background levels 12 h and 24 h after exposure to 5–20 μg/ml of elemene emulsion (data not shown). At 36 h, pronounced apoptotic cell death was observed following exposure to 10–20 μg/ml of elemene emulsion (Figure 1A). Morphological evaluation of apoptosis using Hoechst 33342 reinforced these observations. The elemene emulsion-induced apoptosis was further confirmed by caspase-3 activity assay. After cells were incubated for 36 h with different concentrations of elemene emulsion, a striking increase in caspase-3 activity was observed in A549 cells (Figure 1B). 10 μg/ml and 20 μg/ml elemene emulsion induced ≈2.1-fold and ≈5.2-fold increases compared to control, respectively.

Disruption of lysosomal membrane and decrease of mitochondrial membrane potential

Elemene emulsion-induced lysosomal damage was assessed by determination of increased cytosolic/nuclear AO fluorescence in A549 cells. As early as 12 h following exposure to 20 μg/ml of elemene emulsion, A549 cells had increased fluorescence, indicating the change of localization of AO from lysosomes to the cytoplasm (Figure 2). The lysosomal alternations were persistent, as shown by measurement of AO-fluorescence in cells exposed to elemene emulsion for 24 h and 36 h. In spite of the increased AO fluorescence, A549 cells treated with elemene emulsion for 12 h exhibited mitochondrial membrane potential similar to control cells (Figure 3). After exposure to elemene emulsion for 24 h, the decrease in mitochondrial membrane potential was detected, and this depolarization was more dramatic after 36 h. These results showed that LMP and decrease of mitochondrial membrane potential occurred following elemene emulsion exposure with lysosome changes preceding mitochondrial membrane potential changes.

Western bolts analysis of cathepsin D

To identify the possible involvement of the lysosomal systems, we sought to further characterize cathepsin D protein expression. A549 cells were exposed to different concentrations of elemene emulsion for 12 h. Following treatment with elemene emulsion, there was a clear increase in cathepsin D expression in A549 cells (Figure 4). It is possible to infer that cathepsin D may play an important role in the induction of apoptosis by elemene emulsion exposure.

Generation of ROS and depletion of intracellular GSH

To identify the role of oxidative stress as a potential regular of elemene emulsion-induced apoptosis, we monitored the levels of intracellular ROS and GSH, respectively. As shown in Figure 5 and Figure 6, there were no significant changes concerning GSH and

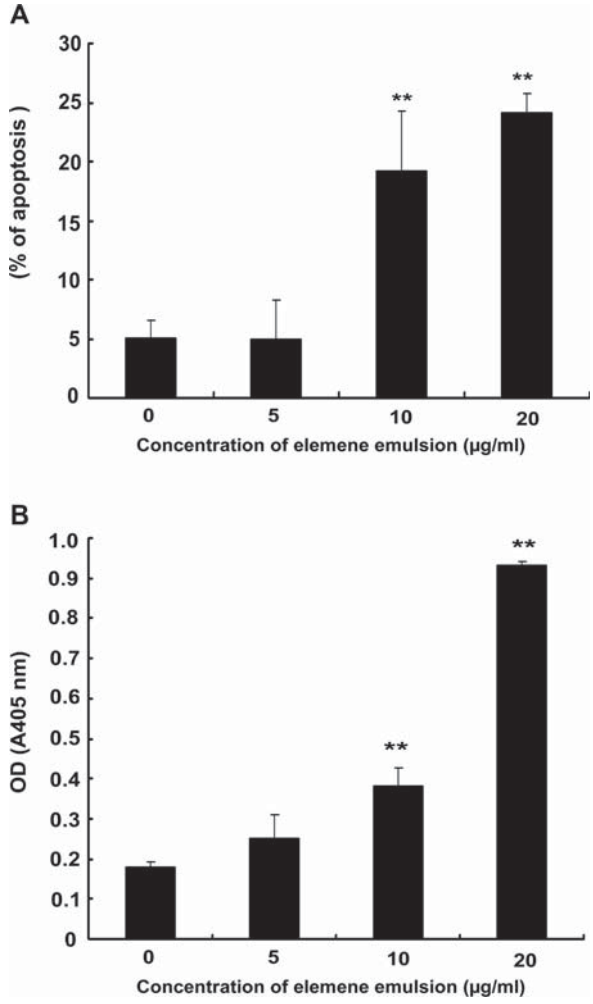

Figure 1. Evaluation of cell death and activation of caspase-3 in A549 cells after elemene emulsion treatment. Cells were exposed to 0–20 μg/ml of elemene emulsion for 36 h. (A) A549 cells were stained with a combination of Annexin V and PI to assay for viable, apoptotic, and necrotic cells. Fluorescence intensities were measured by flow cytometry. (B) Caspase-3 activity of elemene emulsion-incubated A549 cells was measured as the amount of pNA liberated from Ac-DEVD −pNA after a 4h incubation at 37°C, using a microplate reader at 405 nm. The illustrate results are mean ± S.D. of three independent experiments (n = 3). $^{**}P < 0.01$ vs control.

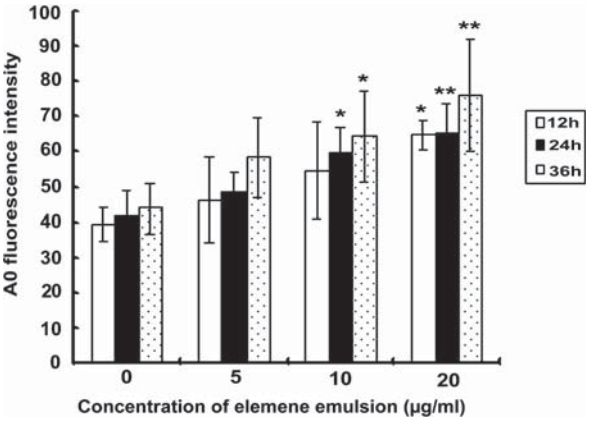

Figure 2. Evaluation of elemene emulsion-induced LMP in A549 cells. Cells were incubated with various concentrations of elemene emulsion for 12 h, 24 h, and 36 h. LMP was analyzed with a fluorescence spectrophotometer using AO. The illustrate results are mean ± S.D. of three independent experiments (n = 3). $^{*}P < 0.05$ vs control. $^{**}P < 0.01$ vs control.

ROS levels when A549 cells were exposed to elemene emulsion for 12 h. A significant increase of DCF fluorescence intensity was observed in cells treated with elemene emulsion for 24 h (Figure 5). We also determined GSH level under the same experimental conditions. The cellular thiols are endogenous antioxidants that can counteract ROS. After cells were incubated with elemene emulsion for 24 h, a striking decrease of intracellular GSH was observed (Figure 6).

Induction of DNA damage

The ability of elemene emulsion to cause DNA damage was assessed with the Comet assay. Under alkaline

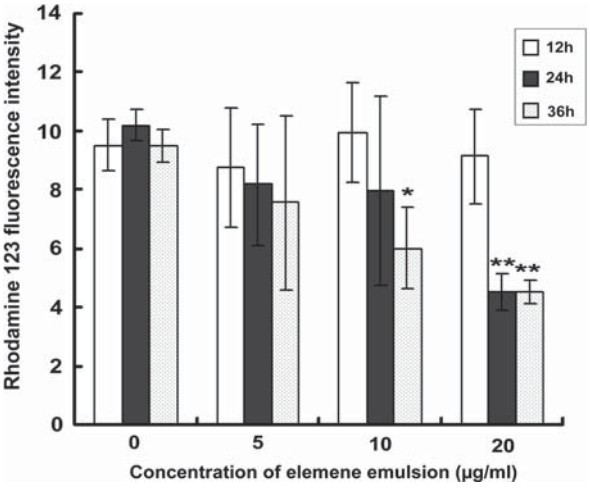

Figure 3. Decrease of mitochondrial membrane potential induced by elemene emulsion in A549 cells. A549 cells were exposed to 0–20 μg/ml of elemene emulsion for 12 h, 24 h, and 36 h, and the mitochondrial membrane potential was assayed using the probe rhodamine 123. The illustrate results are mean ± S.D. of three independent experiments (n = 3). $^{*}P < 0.05$ vs control. $^{**}P < 0.01$ vs control.

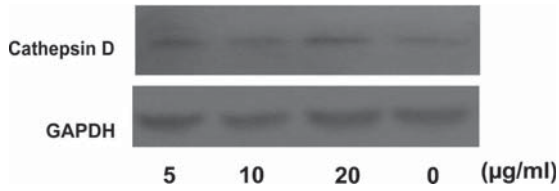

Figure 4. Elemene emulsion induced cathepsin D activation in A549 cells. Cells were incubated with various concentrations of elemene emulsion for 12 h. A dose course of cathepsin D translocation into cytoplasm was investigated by western blot analysis. Following treatment with elemene emulsion, there was a clear increase in cathepsin D expression in A549 cells.

conditions, it detects DNA single- and double-strand breaks and alkali-labile sites. As shown in Figure 7, DNA damage was induced in A549 cells from 24 h treatment of elemene emulsion, which is convinced by the increased quantitative comet tail moment.

Cathepsin D contributed to elemene emulsion-induced apoptosis

To assess the role of cathepsin D in elemene emulsion-induced apoptosis, an inhibitor for cathepsin D (pepstatin A) was used to investigate its involvement with DNA damage (24 h), mitochondrial membrane potential (24 h), caspase-3 activation, and apoptosis (36 h) following 20 μg/ml of elemene emulsion exposure. Figure 8A shows that elemene emulsion elicited a 20-fold increase of comet tail moment and that pepstatin A significantly reduced this effect. As shown in Figure 8B, inhibition of cathepsin D with pepastatin A significantly attenuated elemene emulsion-induced decrease in mitochondrial membrane potential. Pepstatin A treatment also inhibited activation of caspase-3 in A549 cells

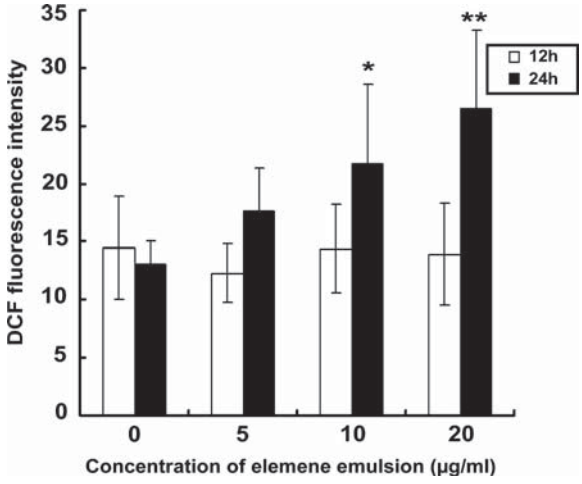

Figure 5. Detection of elemene emulsion-elicited cellular ROS in A549 cells. Cellular ROS was assayed using a fluorescence spectrophotometer (DCFH-DA staining) following administration of elemene emulsion for 24 h. Fluorescence intensities are presented as mean ± S.D. of three independent experiments (n = 3). $^{*}P < 0.05$ vs control. $^{**}P < 0.01$ vs control.

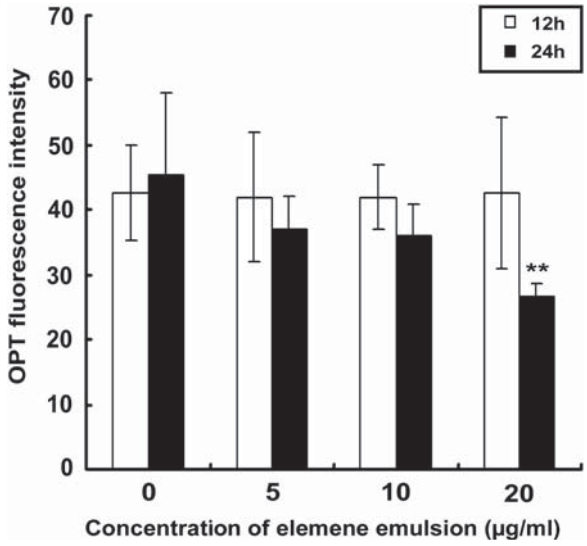

Figure 6. Reduction of intracellular GSH caused by elemene emulsion. After 24 h exposure, the intracellular GSH was measured spectrophotometrically following incubation with OPT. Data presented as mean ± S.D. of three independent experiments (n = 3). $^{**}P < 0.01$ vs control.

following exposure to 20 μg/ml elemene emulsion for 36h (Figure 8C). In addition, elemene emulsion-induced apoptosis in A549 cells was inhibited by pepstatin A, as determined by flow cytometry using Annexin V and PI (Figure 8D). These results provide evidence that inhibition of cathepsin D prevent apoptosis induced by elemene emulsion.

Discussion

Here we investigate the molecular mechanisms of elemene emulsion-triggered apoptosis. As early as 12 h following exposure to elemene emulsion, A549 cells had increased AO fluorescence, indicating the change of localization of AO from lysosomes to the cytoplasm. Lysosomes could be one of the main biochemical targets for elemene emulsion in A549 cells since the lysosomal damage was found to precede any effect on mitochondrial, GSH levels, formation of ROS, DNA, or apoptosis. Involvement of lysosomes and lysosomal proteases, also known as cathepsins, has been now established in a number of cell models as one of the important pathways in apoptosis [33,34]. Cathepsins are members of the papain superfamily of cysteine proteases [35], and predominantly located in lysosomes. Because cathepins B, L, and D are abundant among the lysosomal proteases, they are often used as markers of lysosomal activation [36]. Cathepsin B appears to be involved in TNF-induced apoptosis [37], whereas cathepsin D contributes to apoptosis induced by oxidative stress [38], TNF-α [39], Fas [40], or free radical injury [41]. In the present study, a significant increase in protein expression for cathepsin D was observed utilizing Western blot analysis.

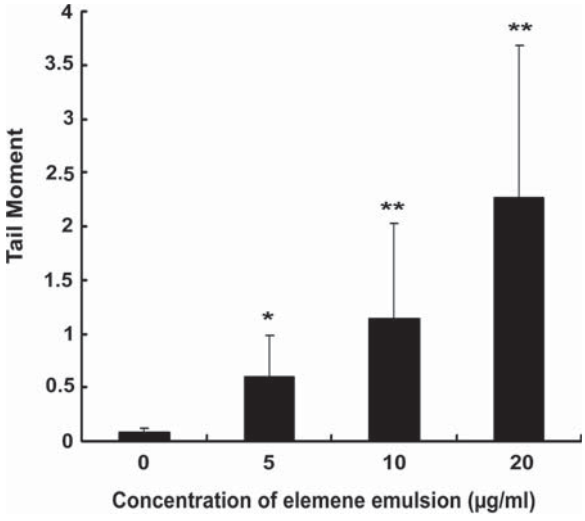

Figure 7. Increase of comet tail moment following elemene emulsion treatment in A549 cells. The alkaline Comet assay was applied to evaluate DNA damage associated with exposure to elemene emulsion for 24 h. Results are indicated as mean ± S.D. of three independent experiments (n=3). $^{*}P < 0.05$ vs control. $^{**}P < 0.01$ vs control.

Oxidative stress refers to a serious imbalance between production of reactive species and antioxidant defence [42]. To explore the role of oxidative stress in elemene emulsion-induced apoptosis, we tested the effects of elemene emulsion on the formation of intracellular ROS and cellular GSH content in A549 cells. Here we found a dose-dependent ROS production and depletion of cellular GSH following a 24 h exposure of cells to elemene emulsion. ROS have been suggested as possible mediators of apoptosis induced by δ-elemene and N-(β-elemene-13-yl) tryptophan methyl ester [43–45]. Mitochondrial are one of the most important cellular sources of ROS production and are particularly susceptible to oxidative stress [46]. The release of lysosomal enzymes into the cell cytoplasm may directly and/or indirectly cause enhanced formation of mitochondrial ROS and these ROS of mitochondrial origin could promote further lysosomal rupture [47]. GSH is the major intracellular antioxidant, which serves as a substrate for GSH peroxidase to degrade hydrogen peroxide to H_2O and also acts as a free radical scavenger [48]. In the present study, a decrease of intracellular GSH was observed in A549 cells exposed to elemene emulsion.

The Comet assay is a sensitive test for the measurement of DNA damage in individual cells. We detected single- and double-strand breaks and/or alkali-labile sites in DNA after exposure of A549 cells to elemene emulsion using Comet assay. The degrees of DNA damage were augmented as the concentrations of elemene emulsion increased. When the damage reaches to a certain level, cell apoptosis or death would eventually occur. DNA damage can be either the consequence of a direct interaction of

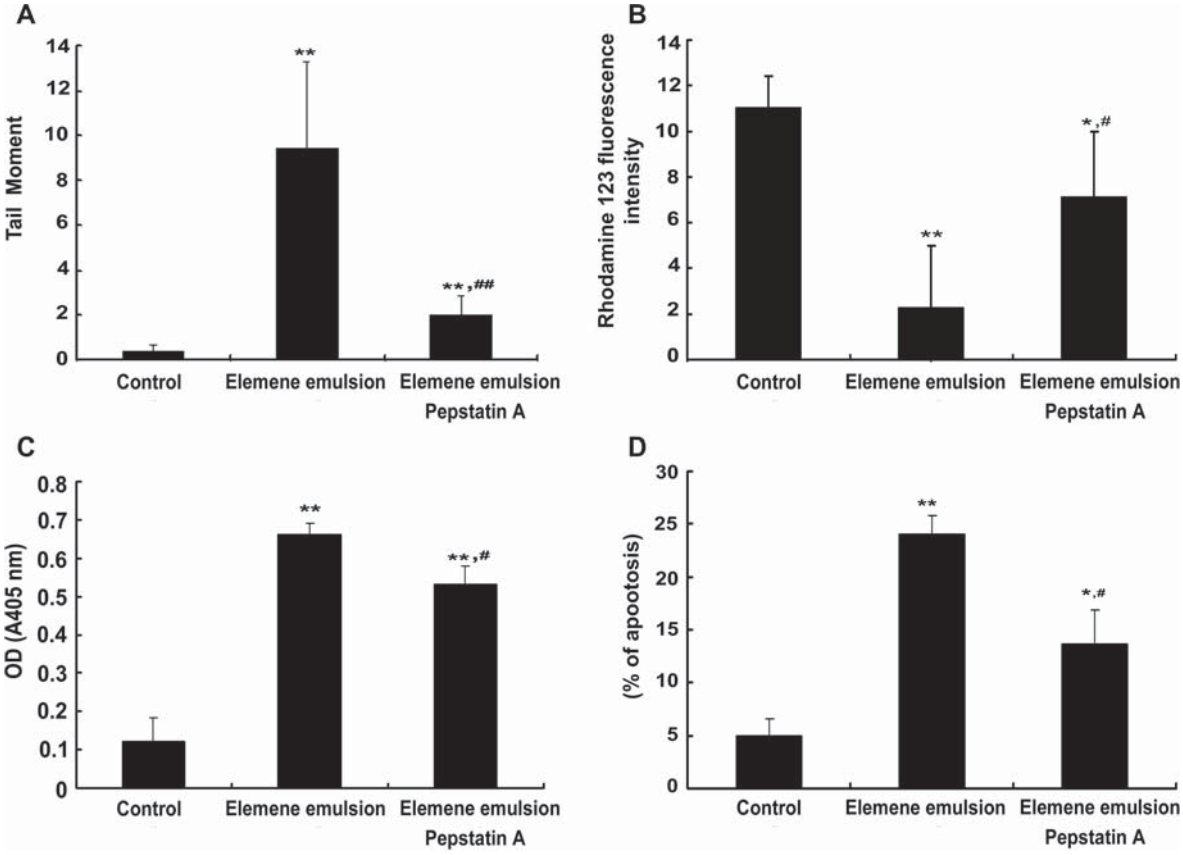

Figure 8. The effect of cathepsin D inhibitor (pepstatin A, 50 μM) on A549 cells 24 h or 36 h following exposure to 20 μg/ml elemene emulsion. A549 cells were pretreated 24 h with or without 50 μM pepstatin A, then treated concomitantly with either media or elemene emulsion for 24 h or 36 h. Treatment of A549 cells with pepstatin A demonstrated a significant inhibition in (A) comet tail moment after exposure to elemene emulsion for 24 h, (B) decrease of mitochondrial membrane potential in A549 cells exposed to elemene emulsion for 24 h, (C) caspase-3 activity in A549 cells treated with elemene emulsion for 36 h, (D) percentage of apoptotic cells exposed to elemene emulsion for 36 h. Results are indicated as mean ± S.D. of three independent experiments (n = 3). $^{*}P < 0.05$ vs control, $^{**}P < 0.01$ vs control. $^{\#}P < 0.05$ vs elemene emulsion alone, $^{\#\#}P < 0.01$ vs elemene emulsion alone.

elemene emulsion with DNA or indirectly due to the generation of ROS. Lysosomal enzymes can target the cell membrane and proteins whereas the release of DNA-ase can induce DNA double strand breaks [49]. DNA damage is signaled to the mitochondrial and cause apoptosis through multiple independent pathway [50].

It is widely accepted that perturbations in the mitochondrial contribute to apoptosis. To establish whether mitochondrial damage is involved in elemene emulsion-induced apoptosis in A549 cells, we assessed changes in the mitochondrial membrane potential by using the cationic dye rhodamine 123. In this work, the mitochondrial membrane potential was found significantly decreased after exposure to elemene emulsion for 24 h, that is, several hours after the lysosomes had begun to undergo leakage. This suggests the existence of a lysosomal-mitochondrial crosstalk associated with elemene emulsion-induced apoptosis. A possible mechanism by which lysosomal protease exerts its effect is by cleavage and activation of Bid (a full-length, inactive Bcl-2 related protein) to a proapoptotic truncated protein (tBid), as a final result of mitochondrial depolarization and cytochrome c release [51–53]. Along these lines, cathepsin D has been suggested to mediate mitochondrial apoptotic pathway by activation of Bid [54]. The studies of Bidere et al. [55] suggest that cathepsin D may activate Bax in a Bid-independent manner as demonstrated in staurosporine-treated T cells. Also significant is that cathepsins can mediate the activation of phospholipase A2 (PLA2), which may attack both mitochondrial and lysosomal membranes and causing further lysosomal rupture [56].

We found that mitochondrial dysfunction and caspase activation were involved in the apoptotic process caused by elemene emulsion, which is consistent with previous investigations [15–17]. Our results also suggest that elemene emulsion-induced apoptosis depends in part on the lysosomes. There may be a direct activation of caspase by cathepsin B [57]. Cathepsins D and L have also been shown to directly activate caspase in some cell models [58,59]. In our present study, the cathepsin D inhibitor pepstatin A prevented subsequent DNA damage, decrease of mitochondrial membrane potential, caspase-3 activation, and apoptosis, which suggests that the release of cathepsin D from disrupted lysosomes

plays an important role in elemene emulsion-induced apoptosis in A549 cells.

In conclusion, our data suggest that LMP associated with relocation of cathepsin D play important and initiating roles during elemene emulsion-induced apoptosis in A549 cells and that precede decrease of mitochondrial membrane potential, oxidative stress, DNA damage, caspase activation, and apoptosis.

Declaration of Interest

The authors declare that they have no conflicts of interest. This study was supported by grants from the National Natural Science Foundation of China (No. 30770646) and Education Committee Foundation of Liaoning Province (2008T031).

References

[1] Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. Int J Cancer 2001;94: 153–156.

[2] Beadsmoore CJ, Screaton NJ. Classification, staging and prognosis of lung cancer. Eur J Radiol 2003;45:8–17.

[3] Molina JR, Yang P, Cassivi SD, Schild SE, Adjei AA. Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. Mayo Clin Proc 2008;83:584–594.

[4] Ciardiello F, Tortora G. A novel approach in the treatment of cancer. targeting the epidermal growth factor receptor. Clin Cancer Res 2001;7:2958–2970.

[5] Li WY, Chan SW, Guo DJ, Chung MK, Leung TY, Yu PH. Water extract of Rheum officinale Baill induces apoptosis in human lung adenocarcinoma A549 and human breast cancer MCF-7 cell lines. J Ethnopharmacol 2009;124:251–256.

[6] Guo YT. Isolation and identification of elemene from the essential oil of Curcuma wenyujin, Zhong Yao Tong Bao 1983;8:31–34.

[7] Yao YQ, Ding X, Jia YC, Huang CX, Wang YZ, Xu YH. Anti-tumor effect of beta-elemene in glioblastoma cells depends on p38 MAPK activation. Cancer Lett 2008;264:127–134.

[8] Zhu T, Zhao Y, Zhang J, Li L, Zou L, Yao Y, Xu Y. ß-Elemene inhibits proliferation of human glioblastoma cells and causes cell-cycle G0/G1 arrest via mutually compensatory activation of MKK3 and MKK6. Int J Oncol 2011;38(2):419–426.

[9] Yuan J, Gu ZL, Chou WH, Kwok CY. Elemene induces apoptosis and regulates expression of bcl-2 protein in human leukemia K562 cells. Zhongguo Yao Li Xue Bao 1999;20: 103–106.

[10] Yang H, Wang X, Yu L. The antitumor activity of elemene is associated with apoptosis. Zhonghua Zhong Liu Za Zhi 1996; 18:169–172.

[11] Zou L, Liu W, Yu L. beta-elemene induces apoptosis of K562 leukemia cells. Zhonghua Zhong Liu Za Zhi 2001;23: 196–198.

[12] Tao L, Zhou L, Zheng L, Yao M. Elemene displays anti-cancer ability on laryngeal cancer cells in vitro and in vivo. Cancer Chemother Pharmacol 2006;58:24–34.

[13] Li X, Wang G, Zhao J, Ding H, Cunningham C, Chen F, et al. Antiproliferative effect of beta-elemene in chemoresistant ovarian carcinoma cells is mediated through arrest of the cell cycle at the G2-M phase. Cell Mol. Life Sci. 2005;62:894–904.

[14] Hu J, Jin W, Yang PM. Reversal of resistance to adriamycin in human breast cancer cell line MCF-7/ADM by beta-elemene. Zhonghua Zhong Liu Za Zhi 2004;26:268–270.

[15] Wang G, Li X, Huang F, Zhao J, Ding H, Cunningham C, et al. Antitumor effect of beta-elemene in non-small-cell lung cancer cells is mediated via induction of cell cycle arrest and apoptotic cell death. Cell Mol Life Sci 2005;62:881–893.

[16] Zhao J, Li QQ, Zou B, Wang G, Li X, Kim JE, et al. In vitro combination characterization of the new anticancer plant drug beta-elemene with taxanes against human lung carcinoma. Int J Oncol 2007;31:241–252.

[17] Li QQ, Wang G, Zhang M, Cuff CF, Huang L, Reed E. beta-Elemene, a novel plant-derived antineoplastic agent, increases cisplatin chemosensitivity of lung tumor cells by triggering apoptosis. Oncol Rep 2009;22:161–170.

[18] Logue SE, Martin SJ. Caspase activation cascades in apoptosis. Biochem Soc Trans 2008;36:1–9.

[19] Li Z, Berk M, McIntyre TM, Gores GJ, Feldstein AE. The lysosomal-mitochondrial axis in free fatty acid-induced hepatic lipotoxicity. Hepatology 2008;47:1495–1503.

[20] Kirkegaard T, Jäättelä M. Lysosomal involvement in cell death and cancer. Biochim Biophys Acta 2009;1793:746–754.

[21] Daré E, Li W, Zhivotovsky B, Yuan X, Ceccatelli S. Methylmercury and H_2O_2 provoke lysosomal damage in human astrocytoma D384 cells followed by apoptosis. Free Radic Biol Med 2001;30:1347–1356.

[22] Larsson DA, Baird S, Nyhalah JD, Yuan XM, Li W. Oxysterol mixtures, in atheroma-relevant proportions, display synergistic and proapoptotic effects. Free Radic Biol Med 2006;41: 902–910.

[23] Erdal H, Berndtsson M, Castro J, Brunk U, Shoshan MC, Linder S. Induction of lysosomal membrane permeabilization by compounds that activate p53-independent apoptosis. Proc Natl Acad Sci U S A 2005;102:192–197.

[24] Zdolsek J, Zhang H, Roberg K, Brunk U. H_2O_2-mediated damage to lysosomal membranes of J-774 cells. Free Radic Res Commun 1993;18:71–85.

[25] Werneburg NW, Guicciardi ME, Bronk SF, Gores GJ. Tumor necrosis factor-alpha-associated lysosomal permeabilization is cathepsin B dependent. Am J Physiol Gastrointest Liver Physiol 2002;283:G947–G956.

[26] Brunk UT, Svensson I. Oxidative stress, growth factor starvation and Fas activation may all cause apoptosis through lysosomal leak. Redox Rep 1999;4:3–11.

[27] De Duve C, de Barsy T, Poole B, Trouet A, Tulkens P, Van Hoof F. Commentary. Lysosomotropic agents. Biochem Pharmacol 1974;23:2495–2531.

[28] Pourahmad J, O'Brien PJ, Jokar F, Daraei B. Carcinogenic metal induced sites of reactive oxygen species formation in hepatocytes. Toxicol In Vitro 2003;17:803–810.

[29] Singh NP, Stephens RE. Microgel electrophoresis: sensitivity, mechanisms, and DNA electrostretching. Mutat Res 1997; 383:167–175.

[30] Sohn JH, Han KL, Lee SH, Hwang JK. Protective effects of panduratin A against oxidative damage of tert-butylhydroperoxide in human HepG2 cells. Biol Pharm Bull 2005;28:1083–1086.

[31] LeBel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 1992;5:227–231.

[32] Hissin PJ, Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 1976;74:214–226.

[33] Turk B, Stoka V, Rozman-Pungercar J, Cirman T, Droga-Mazovec G, Oresić K, Turk V. Apoptotic Pathways: involvement of lysosomal proteases. BiolChem 2002;383:1035–1044.

[34] Kroemer G, Jäättelä M. Lysosomes and autophagy in cell death control. Nat Rev Cancer 2005;5:886–897.

[35] Calkins CC, Sameni M, Koblinski J, Sloane BF, Moin K. Differential localization of cysteine protease inhibitors and a target cysteine protease, cathepsin B, by immuno-confocal microscopy. J Histochem Cytochem 1998;46:745–751.

[36] Ghavami S, Eshragi M, Ande SR, Chazin WJ, Klonisch T, Halayko AJ, et al. S100A8/A9 induces autophagy and apoptosis

via ROS-mediated cross-talk between mitochondria and lysosomes that involves BNIP3. Cell Res 2010;20:314–331.
[37] Foghsgaard L, Wissing D, Mauch D, Lademann U, Bastholm L, Boes M, et al. Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. J Cell Biol 2001;153:999–1010.
[38] Kågedal K, Johansson U, Ollinger K. The lysosomal protease cathepsin D mediates apoptosis induced by oxidative stress. FASEB J 2001;15:1592–1594.
[39] Démoz M, Castino R, Cesaro P, Baccino FM, Bonelli G, Isidoro C. Endosomal-lysosomal proteolysis mediates death signalling by TNFalpha, not by etoposide, in L929 fibrosarcoma cells: evidence for an active role of cathepsin D. Biol Chem 2002;383:1237–1248.
[40] Deiss LP, Galinka H, Berissi H, Cohen O, Kimchi A. Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. EMBO J 1996;15:3861–3870.
[41] Ollinger K. Inhibition of cathepsin D prevents free-radical-induced apoptosis in rat cardiomyocytes. Arch Biochem Biophys 2000;373:346–351.
[42] Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol 2004;142: 231–255.
[43] Wang XS, Yang W, Tao SJ, Li K, Li M, Dong JH, Wang MW. Effect of delta-elemene on Hela cell lines by apoptosis induction. Yakugaku Zasshi 2006;126:979–990.
[44] Xie CY, Yang W, Li M, Ying J, Tao SJ, Li K, et al. Cell apoptosis induced by delta-elemene in colorectal adenocarcinoma cells via a mitochondrial-mediated pathway. Yakugaku Zasshi 2009;129:1403–1413.
[45] Yu Z, Wang R, Xu L, Dong J, Jing Y. N-(beta-Elemene-13-yl) tryptophan methyl ester induces apoptosis in human leukemia cells and synergizes with arsenic trioxide through a hydrogen peroxide dependent pathway. Cancer Lett 2008; 269:165–173.
[46] Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med 2000; 29:222–230.
[47] Zhao M, Antunes F, Eaton JW, Brunk UT. Lysosomal enzymes promote mitochondrial oxidant production, cytochrome c release and apoptosis. Eur J Biochem 2003;270: 3778–3786.
[48] Nappi AJ, Vass E. Comparative studies of enhanced iron-mediated production of hydroxyl radical by glutathione, cysteine, ascorbic acid, and selected catechols. Biochim Biophys Acta 1997;1336:295–302.
[49] Bradley MO, Taylor VI, Armstrong MJ, Galloway SM. Relationships among cytotoxicity, lysosomal breakdown, chromosome aberrations, and DNA double-strand breaks. Mutat Res 1987;189:69–79.
[50] Norbury CJ, Zhivotovsky B. DNA damage-induced apoptosis. Oncogene 2004;23:2797–2808.
[51] Stoka V, Turk B, Schendel SL, Kim TH, Cirman T, Snipas SJ, et al. Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. J Biol Chem 2001;276:3149–3157.
[52] Reiners JJ Jr, Caruso JA, Mathieu P, Chelladurai B, Yin XM, Kessel D. Release of cytochrome c and activation of pro-caspase-9 following lysosomal photodamage involves Bid cleavage. Cell Death Differ 2002;9:934–944.
[53] Cirman T, Oresi K, Mazovec GD, Turk V, Reed JC, Myers RM, et al. Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins. J Biol Chem 2004;279:3578–3587.
[54] Heinrich M, Neumeyer J, Jakob M, Hallas C, Tchikov V, Winoto-Morbach S, et al. Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation. Cell Death Differ 2004;11:550–563.
[55] Bidère N, Lorenzo HK, Carmona S, Laforge M, Harper F, Dumont C, Senik A. Cathepsin D triggers Bax activation, resulting in selective apoptosis-inducing factor (AIF) relocation in T lymphocytes entering the early commitment phase to apoptosis. J Biol Chem 2003;278:31401–31411.
[56] Zhao M, Brunk UT, Eaton JW. Delayed oxidant-induced cell death involves activation of phospholipase A2. FEBS Lett 2001;509:399–404.
[57] Vancompernolle K, Van Herreweghe F, Pynaert G, Van de Craen M, De Vos K, Totty N, Sterling A, Fiers W, Vandenabeele P, Grooten J. Atractyloside-induced release of cathepsin B, a protease with caspase-processing activity. FEBS Lett 1998;438:150–158.
[58] Baumgartner HK, Gerasimenko JV, Thorne C, Ashurst LH, Barrow SL, Chvanov MA, et al. Caspase-8-mediated apoptosis induced by oxidative stress is independent of the intrinsic pathway and dependent on cathepsins. Am J Physiol Gastrointest Liver Physiol 2007;293:G296–G307.
[59] Conus S, Perozzo R, Reinheckel T, Peters C, Scapozza L, Yousefi S, Simon HU. Caspase-8 is activated by cathepsin D initiating neutrophil apoptosis during the resolution of inflammation. J Exp Med 2008;205:685–698.

This paper was first published online on Early Online on 16 August 2011.