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Safrole oxide induces apoptosis by up-regulating Fas and FasL instead of integrin β4 in A549 human lung cancer cells

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Abstract—Previously, we found that 3,4-(methylenedioxy)-1-(2′,3′-epoxypropyl)-benzene (safrole oxide) induced a typical apoptosis in A549 human lung cancer cells by activating caspase-3, -8, and -9. In this study, we further investigated which upstream pathways were activated by safrole oxide during the apoptosis. Immunofluorescence assay combined with laser scanning confocal microscopy revealed that both Fas and Fas ligand (FasL) were up-regulated by the small molecule. In addition, Fas protein distribution was altered, showing a clustering distribution instead of a homogeneous one. Subsequently, Western blot analysis confirmed the up-regulations of Fas and its membrane-binding form of FasL (m-FasL), as well as P53 protein. Conversely, safrole oxide hardly affected integrin β4 subunit expression or distribution, which was reflected from the data obtained by immunofluorescence assay combined with laser scanning confocal microscopy. The results suggested that Fas/FasL pathway might be involved in safrole oxide-induced apoptosis of A549 cells, while integrin β4 might be irrelevant to the apoptosis. Nevertheless, we first found the strong expression of integrin β4 in A549 cells. The study first suggested that safrole oxide might be used as a small molecular promoter of Fas/FasL pathway to elicit apoptosis in A549 cells, which would lay the foundation for us to insight into the new strategies for lung cancer therapy.

1. Introduction

Apoptosis, or programmed cell death, is an evolutionarily conserved mechanism to eliminate unwanted cells commonly occurring during development, as well as in many physiological and pathologic processes.[1] Dysregulation of apoptotic signaling can play a primary or secondary role in various diseases with insufficient apoptosis leading to, for example, cancer.[2] Therefore, understanding of the basic mechanisms that underlie apoptosis will point to potentially new targets of therapeutic treatment of cancer.[3] Once critical components of the cellular pro-apoptotic machinery are activated, tumor cells may undergo apoptosis. However, it has been known that the poor ability of tumor cells to undergo spontaneous apoptosis is partly due to inactivation of pro-apoptotic machinery. So, activation of apoptotic pathways by chemotherapeutic agents has been one of the considerable ways for cancer therapy. In this regard, a small molecular promoter, which can be used as the 'key' to the target proteins to initiate apoptotic pathways of cancer cells, would raise exciting possibilities for carcinoma therapy in future.

It is encouraging to note that the 'library' of small molecules is a powerful tool for screening specific drugs that target specific proteins indispensable to the survival or proliferation of cancer cells.[4–6] So we screened numerous small molecules in our 'library' and found out safrole oxide, a kind of cell permeable compound.[7–9] Previously, we found that safrole oxide induced a typical apoptosis in A549 cells. Recently, we further found that safrole oxide induced the apoptosis by activating caspase-3, -8, and -9.[10] However, which apoptotic pathways upstream the caspases are activated by the small molecule is not clear. Nevertheless, it is substantiated that CD95/Fas, a member of tumor necrosis factor receptor superfamily, plays an important role in initiating caspase cascade.[11–13] When Fas is activated by its ligand (FasL), agonistic antibody,[14] or P53 protein,[15,16]

Keywords: Safrole oxide; A549 cell apoptosis; Fas; FasL; P53 protein; Integrin β4.
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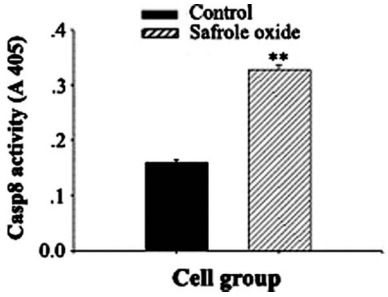

Figure 1. Colorimetric assay for caspase-8 activity at 24 h.[10] Data are means ± SE from two independent experiments. (**$P < 0.01$ vs the control group).

oligomerization and aggregation of Fas receptor occur and result in the assembly of death-induced signaling complex (DISC). Subsequently, caspase-8 is activated and then activates the downstream caspases to initiate apoptosis.[17] Actually, caspase-8 occupies an essential and apical position in Fas signaling pathway.[18] Our previous work showed that caspase-8 was significantly activated in safrole oxide-induced apoptosis of A549 cells (Fig. 1).[10] Besides, Fas can be transcriptionally activated by wild-type p53 (wt p53) in anti-tumor reagents-evoked apoptosis of tumor cells.[19] Coincidently, safrole oxide also up-regulated P53 expression during the apoptosis of A549 cells, which was detected by immunocytochemistry assay previously.[8] Based on these results, we hypothesized that Fas pathway might be activated by safrole oxide in A549 cell apoptosis.

In addition, in some cases, p53 activation in the apoptosis of carcinoma cells is associated with integrin β4, an important subunit of cell adhesion receptor a6β4. Integrin α6β4 is distinguished structurally from other integrins on the basis of the unusually large cytoplasmic domain of its β4 subunit. Some functions of a6β4 can be mediated entirely by integrin β4 cytoplasmic domain.[20] Bachelder et al. reported that exogenous expression of interin β4 gene in α6β4-negative RKO colon cancer cells stimulated apoptosis via α6β4 dependent wt p53 activation.[20] Coincident with this report, Clarke et al. found that overexpression of the cytoplasmic domain of integrin β4 in RKO cells induces apoptosis by the activation of the p21 (WAF/Cip1) pathway.[21] Nevertheless, P21 expression is directly regulated by wt p53.[22] In contrast, in p53-deficient MDA-MB-435 breast cancer cells, exogenous expression of α6β4 plays an anti-apoptotic role by activation of Akt/protein kinase B.[20] Obviously, integrin β4 may activate wt p53 to evoke cell apoptosis. In our previous study, Miao and co-workers showed that integrin β4 mediated apoptotic signal transduction by upregulating p53 protein expression in human vascular endothelial cells.[23] Similarly, in the apoptosis of A549 cells evoked by safrole oxide, the expression of wt P53 was elevated, too.[8] Based on these reports, we hypothesized that integrin β4 might be activated by safrole oxide and then up-regulates P53 to mediate A549 cell apoptosis.

In this work, we investigated the two hypotheses to further understand the apoptosis pathway upstream of caspases, wishing to lay the foundation for finding the specific protein targeted by the small molecule, and wishing to use safrole oxide as a 'key' to the apoptotic pathway to initiate lung cancer cell apoptosis.

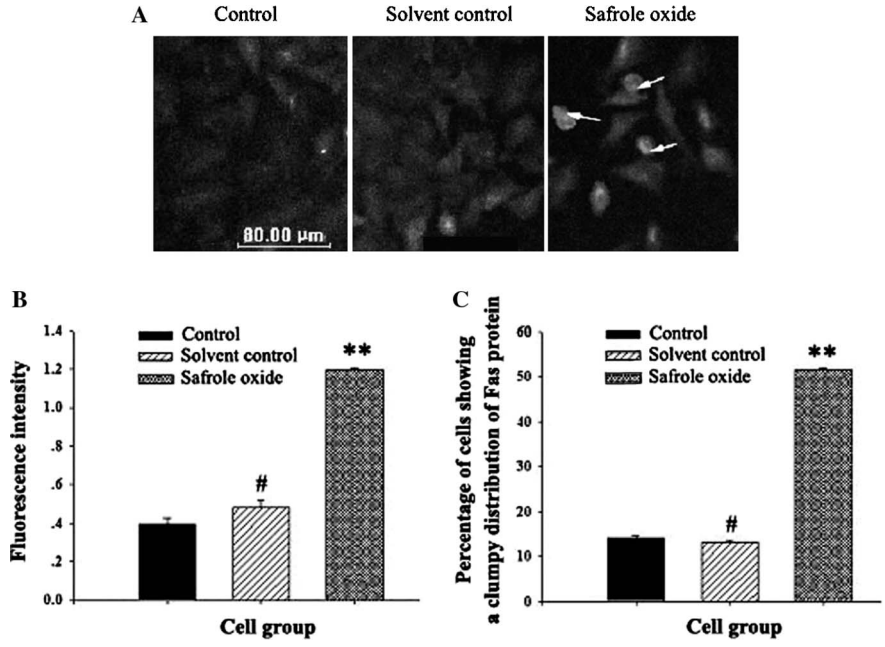

Figure 2. Immunofluorescence assay for Fas expression and localization in A549 cells at 24 h. Cells were treated with or without safrole oxide, 224.72 μM, for 24 h. After fixation, the cells were incubated orderly with blocking serum, monoclonal antibody against Fas, FITC-conjugated secondary antibody, and then fluorescence intensity was quantitatively analyzed by laser scanning confocal microscopy. The expression and localization of Fas protein are shown as photographs (A). The values of relative fluorescence intensity are depicted as a bar chart (B). The percentage of cells showing a clumpy distribution of Fas protein is depicted as a bar chart (C). The experiment was performed independently three times. ($^{\#}P > 0.05$, **$P < 0.01$ vs the control group).

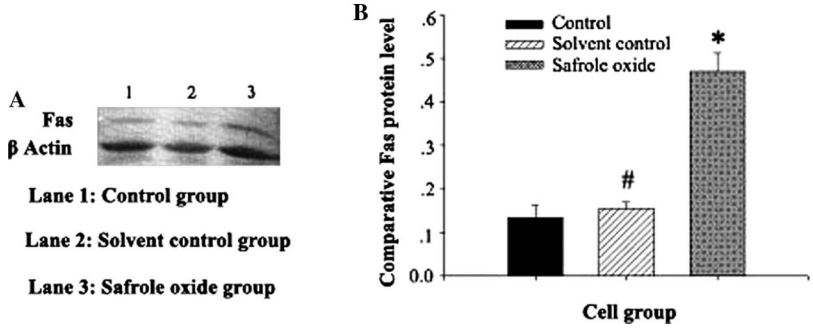

Figure 3. Western blot assay for Fas protein expression in A549 cells at 24 h. Protein extracts were prepared from the cells in the control group, solvent control group, and safrole oxide-treated group. The blots shown on the left are a representative of two independent experiments (A). The comparative level of Fas protein expression was normalized by density ratio of Fas to β-actin on the same lane, depicted as a bar chart on the right (B). ($^{\#}P > 0.05$, $^{*}P < 0.05$ vs the control group).

2. Results

2.1. Effects of safrole oxide on Fas protein expression and localization

In comparison with the control group, the fluorescence intensity, which reflected the relative quantity of Fas protein expression, was enhanced from 0.395 to 1.196 (elevated to 3.03-fold) by safrole oxide ($P < 0.01$, $n = 3$) (Figs. 2A and B). Simultaneously, Fas protein distribution on cell membrane was altered. In the control and the solvent control groups, Fas was diffusely distributed across the cell surface. However, in safrole oxide-treated cells, Fas was amassed into large patches, and some patches were found to migrate toward one pole of the cell and showed cap-like forms (marked by arrows) (Fig. 2A). The percentage of cells showing a patch distribution of Fas protein increased from 13.98% to 51.50% ($P < 0.01$, $n = 3$) (Figs. 2A and C). However, in the solvent control group, ethanol 0.04% (V/V) hardly affected Fas expression and localization ($P > 0.05$, $n = 3$) (Figs. 2A–C). The data suggested that Fas might be activated by the compound.

For accurate quantification, we detected the changes in Fas expression by Western blot assay. In comparison with the control group, Fas expression was up-regulated to 3.48-fold by safrole oxide ($P < 0.05$, $n = 2$) (Fig. 3). The data confirmed that Fas expression was promoted during safrole oxide-induced apoptosis of A549 cells.

2.2. Effects of safrole oxide on the expression of FasL

To determine whether Fas activation was related with FasL in the apoptosis of A549 cells, we subsequently examined the expression of FasL. The bright-field confocal micrographs (Figs. 4A–C) and the immunofluorescence confocal micrographs (Figs. 4D–F) showed that the N-terminus of FasL had a cytoplasmic distribution. The data are consistent with the reports that FasL is type II transmembrane protein of the tumor necrosis factor (TNF) family.[24,25] In addition, compared with the control group, the average fluorescence intensity per cell in the test group was enhanced to 1.90-fold by safrole oxide at 24 h ($P < 0.05$, $n = 3$) (Figs. 4D, F, and G), while there was no significant difference in the fluorescence intensity between the control and the solvent control groups ($P > 0.05$, $n = 3$) (Figs. 4D, E, and G). The data suggested that safrole oxide up-regulated FasL expression in A549 cells at 24 h.

We also examined the changes in FasL expression by Western blot. Anti-FasL polyclonal antibody recognized a 38 kDa band. Because FasL exists in two forms, a membrane-binding form (m-FasL, 37–42 kDa) and a soluble form (s-FasL, 23–26 kDa),[25] the observation of 38 kDa band demonstrated the expression of m-FasL in A549 cells. In comparison with the control group, m-FasL expression was up-regulated to 1.77-fold ($P < 0.05$, $n = 2$) by safrole oxide (Fig. 5). In contrast, ethanol 0.04% (v/v) had hardly contributed to m-FasL up-regulation ($P > 0.05$, $n = 2$) (Fig. 5). The data further revealed that safrole oxide activated FasL (38 kDa) expression in A549 cells.

2.3. Effects of safrole oxide on the expression of P53 protein

Since the activation of wt p53 is able to directly activate Fas,[19] and since safrole oxide-induced up-regulation of wt P53 was previously found by immunocytochemistry assay[8], to understand the relationship between p53 and Fas activation in safrole oxide-induced apoptosis of A549 cells, we further detected the changes in P53 expression by Western blot. Compared with the control group, safrole oxide-treated group (the test group) showed that P53 expression exhibited a 1.13-fold increase (increased to 2.13-fold) ($P < 0.05$, $n = 2$) (Fig. 6). The result confirmed that safrole oxide activated p53 expression in A549 cells at 24 h.

2.4. Effects of safrole oxide on integrin β4 expression and localization

To detect whether integrin β4 was related to wt p53 activation and then mediated safrole oxide-induced apoptosis in A549 cells, we analyzed the changes in the expression and localization of integrin β4 subunit by immunofluorescence assay. In comparison with the control group, the fluorescence intensity, which reflected the expression of integrin β4, was hardly affected by safrole oxide ($P > 0.05$, $n = 3$) (Figs. 7A and B). Additionally,

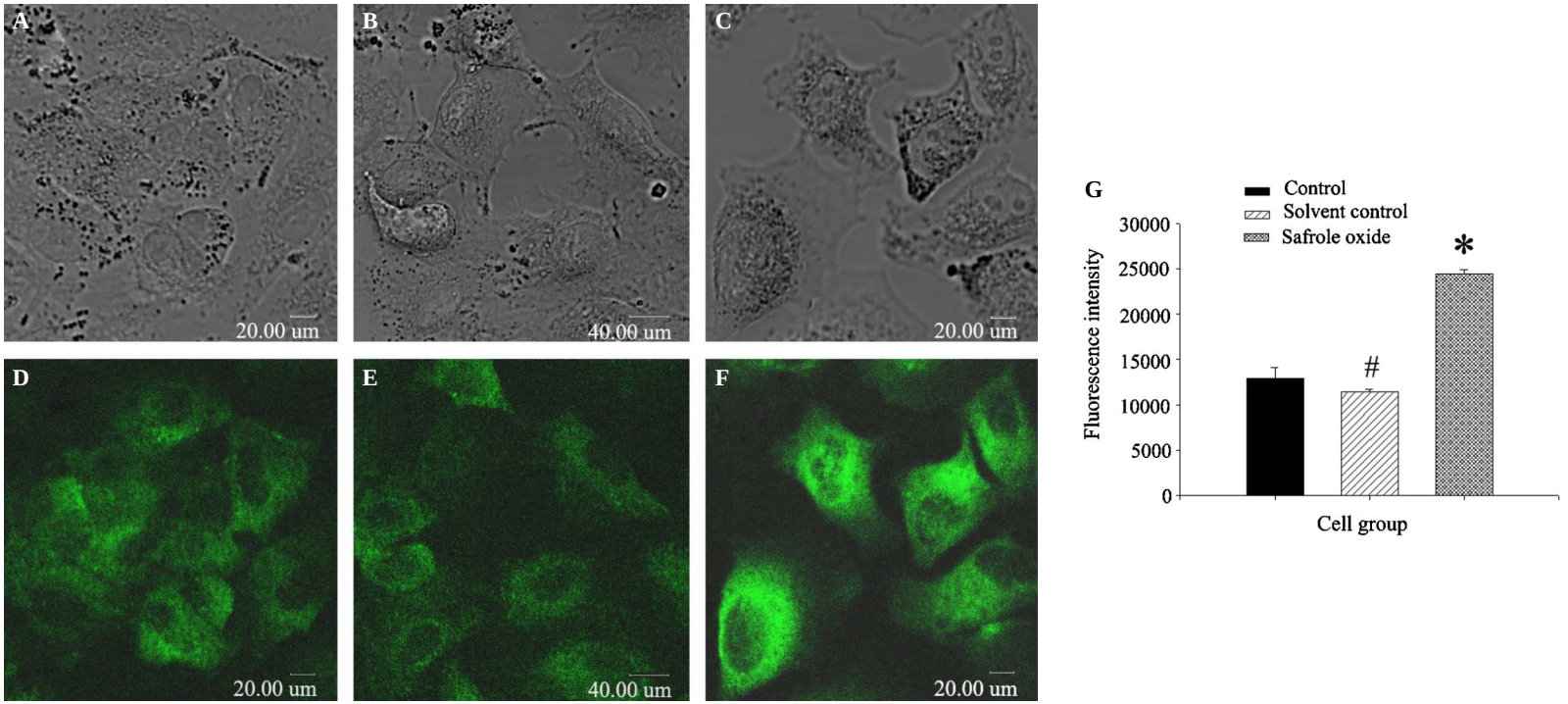

Figure 4. Immunofluorescent assay for FasL expression and localization in A549 cells at 24 h. The changes in FasL protein expression and localization were monitored by immunofluorescence assay as described in Figure 2. (A–C) the bright-field confocal micrographs; (D–F) the immunofluorescence confocal micrographs; (A and D) cells of the control group; (B and E) cells of the solvent control group; (C and F) cells treated by safrole oxide for 24 h; (G) the values of relative fluorescence intensity are depicted as a bar chart. The experiment was performed independently three times. ($^{\#}P > 0.05$, $^{*}P < 0.05$ vs the control group).

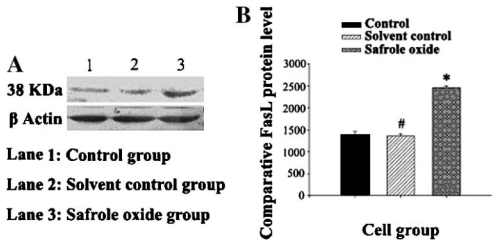

Figure 5. Western blot assay for FasL expression in A549 cells at 24 h. The total protein 50 μg of each sample was separated by a 10% SDS–polyacrylamide gel and immunoblotted. The bands shown on the left (A) are a representative of two independent experiments. The relative quantity of FasL expression is depicted as a bar chart on the right (B). ($^{\#}P > 0.05$, $^{*}P < 0.05$ vs the control group).

no obvious changes in localization were observed (Fig. 7A). The data demonstrated that integrin β4 was not affected by safrole oxide in A549 cell apoptosis. Integrin β4 might not be implicated in the apoptosis pathway induced by the small molecule.

3. Discussion

Previous report shows that synthetic retinoid CD437 promotes Fas expression and induces apoptosis in three human lung cancer cell lines with wt p53.[26] More significantly, our data exhibited that safrole oxide not only up-regulated Fas protein expression but also induced large CD95 patches. Large CD95 clusters suggested oligomerization of a complex including Fas.[27] Recent studies suggest that Fas oligomerization may be critical for optimal signaling.[28] Cremesti et al. showed that in Jurkat cells, cross-linking Fas with anti-Fas IgM CH-11 antibody induced amassing of Fas into patches. Fas patching was rapidly followed by capping. Ultimately, the cells underwent apoptosis. They report that capping of Fas is essential for optimal function in some cells.[28] Apart from anti-Fas antibody, Belka et al. found that Fas ligand induced Fas capping and apoptosis in IL-2-treated and irradiated peripheral blood lymphocytes.[29] Receptor capping may provide the milieu for oligomerization of FADD and caspase-8 to initiate apoptotic response.[28] Excitingly, in this work, both Fas up-regulation and the large CD95 receptor patches elicited by safrole oxide were observed. Some of the patches were found to show cap-like forms. Furthermore, activating caspase-8 is a necessary precondition for Fas pathway activation, and caspase-8 is indeed activated by safrole oxide in the apoptosis of A549 cells.[10] Studies have substantiated that radiation-induced caspase-8 activation and CD95 receptor capping confirm the functional activation of the CD95 receptor.[29] Therefore, our data suggested that Fas pathway might be activated by safrole oxide in A549 cell apoptosis.

Although Fas/FasL interactions result in apoptosis of Fas-expressing cells, remarkably, some anti-tumor re-

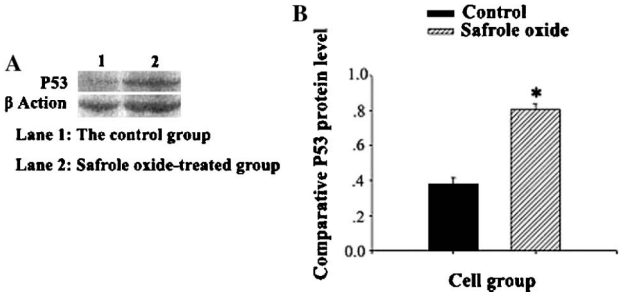

Figure 6. Western blot assay for P53 protein expression in A549 cells at 24 h. Each sample was prepared and examined as described in the experimental section. The blots shown are a representative of two independent experiments (A). The comparative level of P53 protein expression was normalized by the density ratio of P53 to β-actin on the same lane, depicted as a bar chart on the left (B). ($^{*}P < 0.05$ vs the control group).

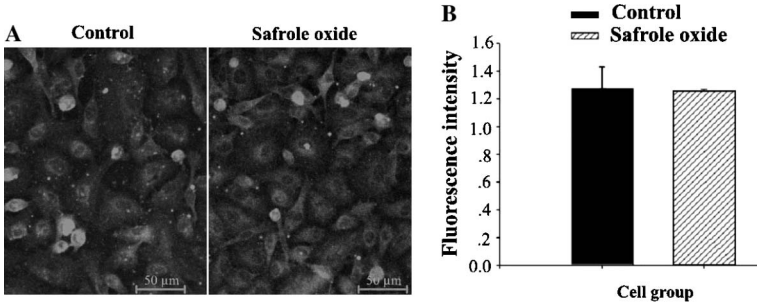

Figure 7. Immunofluorescence assay for integrin β4 expression and localization in A549 cells at 24 h. The changes in the expression and localization of integrin β4 were monitored by immunofluorescence assay as described in Figure 2. The expression and localization of β4 integrin are shown as photographs (A) on the left. The values of fluorescence intensity are depicted as a bar chart (B) on the right. There was no significant difference in β4 integrin expression or localization between the two groups. The experiment was performed independently three times. ($^{\#}P > 0.05$ vs the control group).

agents induce FasL-independent oligomerization of Fas and initiation of Fas-dependent signaling pathways.[16] Whereas, our data showed that safrole oxide promoted m-FasL expression. Some studies report that s-FasL is up to 1000-fold less active than m-FasL in inducing apoptosis,[30,31] while other studies show that both m- and s-FasL binding to the Fas (APO-1/CD95) initiate the apoptotic signal in chemosensitive cells.[32,33] For example, Hsu et al. reported that isoliquiritigenin (ISL), a natural pigment with the simple chalcone structure, enhanced the expression of Fas and its two ligands (m-FasL and sFasL), and the Fas/FasL system may participate in the antiproliferative activity of ISL in A549 cells.[34] In this study, both immunocytochemistry and Western blot evidenced the up-regulation of m-FasL by safrole oxide at 24 h, as well as its receptor Fas. Besides, Fas clustering or oligomerization occurred markedly. These data suggested that Fas/FasL pathway might be activated by safrole oxide and then mediated the apoptosis of A549 cells. Chemotherapy would induce an up-regulation of FasL, leading to an autocrine/paracrine activation of Fas signaling, and this may constitute a potential mechanism in the mediation of anticancer drug-induced apoptosis.[35,36]

Apart from FasL, Fas can also be activated by wt p53 activation. Wt p53 can trigger Fas oligomerization and activate Fas pathway independent of FasL. Some reports substantiate that p53 activation transiently increased surface Fas (CD95) expression by transport from the Golgi complex,[19,37] and then induced clustering of Fas in the same fashion as FasL,[19,29] eventually sensitizing cells to Fas-induced apoptosis. Besides, Muller et al. evidence the crosstalk between Fas expression and p53 in apoptosis.[38] Fas expression can be transactivated by wt p53 through p53-binding sites in the promoter and first intron of the *Fas* gene.[38] So far, it has not been reported whether p53 can not only activate the expression of Fas but also can induce its transportation and oligomerization in the same cell line and by the same stimulus. Our data demonstrated that safrole oxide markedly up-regulated the expression of both Fas and P53, and also showed the induction of oligomerization of Fas during A549 cell apoptosis. These findings revealed the activation of p53 by safrole oxide. We hypothesized that there might be some relationship between the activation of p53 and Fas in safrole oxide-induced apoptosis in A549 cells, which needs further investigation.

p53 can be activated by a number of 'stress' signals, including integrin β4.[20] However, in this work, although safrole oxide elevated wt p53 expression in A549 cell apoptosis, integrin β4 expression and distribution were hardly affected by safrole oxide. The data suggested that integrin β4 might have no contribution to p53 activation in A549 cell apoptosis in response to the small molecule. In contrast, in colon carcinoma HCT116 cells with wild type p53 and a6β4, clustering of a6β4 stimulates p53 activity and cell apoptosis.[20] These contradictory results might be due to the different status of integrin β4. a6β4 expression is frequently altered in neoplastic cells. It is sometimes lost and sometimes overexpressed.[39] Based on these results, we speculated that the normal function of integrin β4 might be disrupted in A549 cells, though it was strongly expressed. This speculation needs to be further investigated.

In summary, these data suggested that safrole oxide, 224.72 μM, triggered apoptotic response in A549 cells at 24 h perhaps through Fas/FasL pathway. However, integrin β4, the subunit of cell adhesion receptor a6β4, might be irrelevant to the programmed cell death. The detailed mechanisms, including how safrole oxide increased the protein levels of Fas, FasL, and P53, need further investigation, but the results first suggested that safrole oxide might be used as a significant promoter of Fas/FasL pathway to initiate A549 human lung cancer cell apoptosis. These findings would lay the foundation for us to further investigate the detailed mechanisms and the other crucial proteins the drug targets.

4. Experimental

4.1. Materials

Safrole (98% (GC)) was purchased from Fluka AG and 3-chloroperoxybenzoic acid from ACROS, Safrole oxide (3,4-(methylenedioxy)-1-(2′,3′-epoxypropyl)-benzene) was synthesized as described by Zhao et al.[40] RPMI 1640 was obtained from Gibco BRL Co. (Grand Island, USA). Newborn calf serum was from Hyclone (USA). Anti-Fas mAb was obtained from Zhongshan Golden Bridge Biotechnology Co. (Beijing, China). Anti-β-actin mAb, rabbit polyclonal antibody against the N-terminus of FasL (anti-FasL Q-20), anti-P53 mAb, anti-β4 antibody sc-9090, FITC-linked goat anti-mouse antibody, FITC-linked goat anti-rabbit antibody, HRP-linked goat anti-mouse antibody, and HRP-linked goat anti-rabbit antibody were all from Santa Cruz Biotech, Santa Cruz, CA. Safrole oxide was dissolved in ethanol and applied to cells such that the final concentration of ethanol in the culture medium was below 0.04% (v/v). Ethanol at a concentration of 0.1% (v/v) did not affect the viability of the cells.

4.2. Cell cultures

A549 lung cancer cells were cultured in RPMI 1640 medium, supplemented with 10% (v/v) newborn calf serum at 37 °C in 5% CO_2, and 95% air. The cells were routinely seeded at the density of 100,000/cm^2 into 96-well plates or other appropriate dishes containing the medium. At 24 h after planting, the experiments given below were performed.

4.3. Immunofluorescent assay for Fas/FasL protein expression and localization

Considering that the drug could initiate apoptosis in A549 cells at the concentrations from 112.36 μM to 449.44 μM,[8] we selected the intermediate concentration (224.72 μM) as a representative to study in this assay and the others below. After the cells were treated with safrole oxide for 24 h, the alterations of Fas and FasL

protein expression and localization were monitored by immunofluorescence assay as described before.[41] Fluorescence intensity was quantitatively analyzed by laser scanning confocal microscope (Leica, Germany).

4.4. Western blotting

Cells were cultured with or without safrole oxide for 24 h. The total protein of the cells was prepared as described by Lipscomb.[42] Protein concentration of each lysate was determined by the Bradford protein assay.[43] Total protein was loaded on 7.5–10% SDS–polyacrylamide gel and electrophoretically transferred to nitrocellulose transfer membrane (Amersham Pharmacia Biotech, USA). After blocking with 5% skim milk in PBS containing 0.5% (v/v) Tween 20 for 1 h, the membrane was incubated with mouse anti-Fas mAb, rabbit anti-FasL polyclonal antibodies Q-20 or mouse anti-P53 mAb overnight at 4 °C, and then incubated with HRP-linked secondary antibodies (goat anti-mouse or goat anti-rabbit IgG) for 1 h at room temperature (rt), followed by color development with 0.06% DAB (diaminobenzidine) and 0.03% H_20_2 in PBS for 3–5 min, rt. Distilled water was used to stop the reaction. The level of β-actin was used as a loading control. The relative quantity of the proteins was analyzed by JEDA software (JEDA com. China). In this assay, cells cultivated in the medium containing ethanol 0.04% (v/v) were used as a solvent control group.

4.5. Immunofluorescent assay for integrin β4 expression

After the cells were treated with safrole oxide 224.72 μM for 24 h, the alteration of integrin β4 protein expression was examined by immunofluorescence assay as described before.[41] Fluorescence intensity was quantitatively analyzed by laser scanning confocal microscope (Carl Zeiss, Germany).

Acknowledgments

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