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LIVE IMAGING OF XIAO-AI-PING-INDUCED CELL DEATH IN HUMAN LUNG ADENOCARCINOMA CELLS

XIAO-PING WANG*,†, TONGSHENG CHEN*,‡,
LONGXIANG WANG* and LEI SUN*

*MOE Key Libratory of Laser Life Science &
Institute of Laser Life Science,
South China Normal University,
Guangzhou 510631, China

†Department of Anesthesiology,
The First Affiliated Hospital of Jinan University,
Guangzhou 510632, China
‡chentsh@scnu.edu.cn

Xiao-Ai-Ping (XAP), a traditional oriental medicinal herb isolated from the stem of Marsdenia tenacissima (Roxb.) Wight et Arn., has been shown to induce tumor cell apoptosis. In this study, we used confocal fluorescence microscopy and fluorescence resonance energy transfer (FRET) techniques to study the molecular mechanism of XAP-induced apoptosis in single living human lung adenocarcinoma (ASTC-a-1) cells. The efficacious apoptosis was observed at 6 h after of 100 μl XAP treatment. Further monitoring the dynamics of caspase-3 activation using FRET imaging in single living ASTC-a-1 cell expressing stably with SCAT3, a FRET probe, showed that XAP activated the caspase-3 at about 2 h after XAP treatment. These data suggest that caspase-3 activation was involved in the XAP-induced apoptosis in ASTC-a-1 cells.

Keywords: Xiao-Ai-Ping (XAP); apoptosis; caspase-3; FRET.

1. Introduction

Apoptosis plays an important role in cell development and homeostasis, and caspase family is crucial in apoptosis,[1] and caspase-3 has been verified as an important executive apoptotic molecule.[2] Xiao-Ai-Ping (XAP), a traditional Chinese medicine isolated from the stem of Marsdenia tenacissima (Roxb) Wight et Arn., has been documented to possess functions against inflammation, asthma and cancer, and is widely employed as a therapeutic drug for tonsillitis, pharygitis, cystitis, pneumonia, asthma and so on.[3,4]

Confocal fluorescence imaging and FRET technology have been widely used to study protein-protein interactions in living cells.[2,5,6] Traditional biophysical and/or biochemical methods can only measure the average behavior of cell populations

‡Corresponding author.

and the static spatial information from fixed cells and thus cannot provide direct access to the interactions of these protein partners in their natural environment.[6] SCAT3 is a FRET indicator of caspase-3 activation,[7] which is composed of enhanced cyan fluorescence protein (ECFP) as the FRET donor and Venus, a variant of enhanced yellow fluorescence protein (EYFP), as the FRET acceptor, linked by peptides containing the caspase-3 cleavage sequence, DEVD.[2,5] This sequence is found in many cytosolic and nuclear caspase substrates and is cleaved by several effector caspases including caspase-3 and caspase-7.[2,7] Caspase-3 is believed to play a central role in the execution of apoptosis, because this enzyme is required for oligonucleosomal DNA fragmentation and promotes the activation of other effector caspases.[2]

The present report used confocal fluorescence imaging and FRET technology based on SCAT3 to study the effect of XAP on caspase-3 activation and cellular morphology in single living human lung adenocarcinoma (ASTC-a-1) cells. A human lung cancer (ASTC-a-1) cell line stably expressing SCAT3[8,9] was used to study caspase-3 activation and cell apoptosis by XAP.

2. Material and Methods

2.1. *Material*

Dulbecco's modified Eagle medium (DMEM) was purchased from GIBCO (Grand Island, NY). Lipofectamine reagent was purchased from Invitrogen (Carlsbad, CA). DNA Extraction kit was purchased from Qiagen (Valencia, CA). SCAT3 was provided by Professor Masayuki Miura.[7] XAP injection was from the Tonghua Shenyuan Phar. Co., Ltd. (State Drug Approval Document No: Z20025869).

2.2. *Cells culture*

ASTC-a-1 cells were grown in DMEM supplemented with 15% fetal calf serum (FCS), and the cells were maintained at 37°C in a humidified atmosphere (95% air and 5% CO_2) at pH 7.4. The constant temperature can be controlled by the temperature-control 37-2 (Carl Zeiss MicroImaging, Inc., Germany).

Plasmid DNA of SCAT3 was transfected into ASTC-a-1 cells by using Lipofectin reagent (Carlsbad, CA). The cells stably expressing SCAT3 reporter were screened with 0.8 mg/ml G418, and positive clones were picked up with micropipettes.[8,9]

2.3. *Cell viability assay*

Cell viability assays were performed by using Cell Counting Kit-8(CCK-8) (WST-8, Dojindo, Kumamoto, Japan), according to the supplier recommendations. Cells were plated in 96-well plates at 5×10^3 cells per well and cultured in the medium with different dose of XAP. At the indicated time points, the cell viability was measured as the absorbance (450 nm) of reduced WST-8. The WST-8 reagent solution (10 μl) was added to each well of a 96 well microplate containing 100 μl of

cells in the culture medium at various densities, and the plate incubated for 2 h at 37°C. Absorbance was measured at 450 nm using auto microplate reader (infinite M200, Tecan, Austria). Cell viability was expressed as the percentage of viable cells relative to untreated cells using the absorbance at 450 nm. All experiments were performed in five wells on three separate occasions.

2.4. *Confocal microscopy and image analysis*

Fluorescence confocal imaging and FRET were performed on a commercial Laser Scanning Microscopes (LSM510/ConfoCor2) combination system with C-Apochromat 40× NA 1.3 and 100 × 1.4 NA oil objective (Carl Zeiss MicroImaging, Inc., Germany). The excitation wavelengths were 458 nm for SCAT3 and 633 nm for Mitotracker Red, The emission fluorescence channels were 470–500 nm bandpass for CFP, 530 nm longpass for Venus, 650 nm longpass for Mitotracker Red. To quantify the results, the images of CFP and Venus emission intensities were processed with Zeiss Rel3.2 image processing software (Carl Zeiss MicroImaging, Inc., Germany).

2.5. *Statistical analyses*

Experiments are means of five plicates, and each experiment was performed five times. Data are expressed as *mean* ± SD. Statistical analyses were performed with SPSS12 (SPSS, Chicago) by using the two-sample t-test. Differences were considered statistically significant when $P \leq 0.05$.

3. Results

3.1. *Inhibition of XAP on cell viability*

The inhibition of XAP with different dose on the survival rate of ASTC-a-1 cells was investigated by a CCK-8 assay. The cells were treated with XAP for 48 h. The results revealed that the XAP decreased the survival rate of ASTC-a-1 cells (Fig. 1), and the $^{*}P$ is less than 0.01 (from 10–50 μl).

3.2. *Effect of XAP on mitochondria*

To study the effect of XAP on the mitochondria, we monitored the dynamics of mitochondria of the cells treated by 40 μl of XAP (Fig. 2). In healthy cells, most of the mitochondria were filar (Fig. 2). However, XAP made the mitochondria became separate dot which seem to be swelling 6 h after XAP treatment (Fig. 2).

3.3. *Effect of XAP on cell morphology*

To determine the effect of XAP on the cell shape, we used the time-lapse confocal microscope to monitor the dynamical changes of cells expressing YFP plasmids after 40 μl of XAP treatment. The YFP distributed evenly in both cytoplasm and

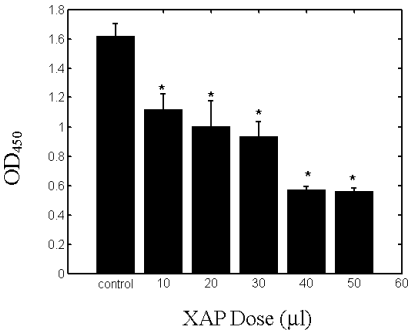


Fig. 1. Inhibition of XAP on the cell viability $*P < 0.01$.

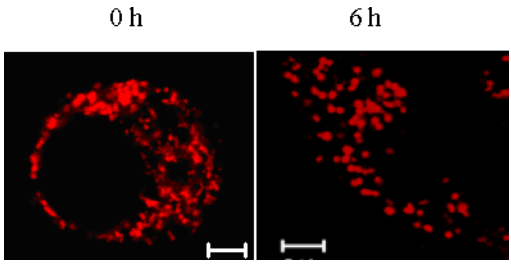


Fig. 2. Effect of XAP on the mitochondria. Bar: 5 μm.

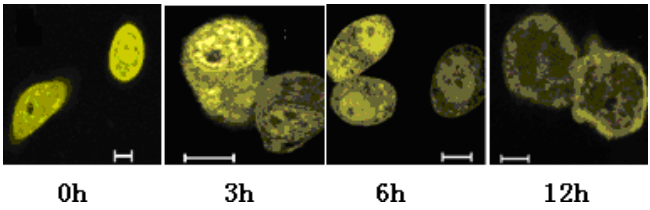
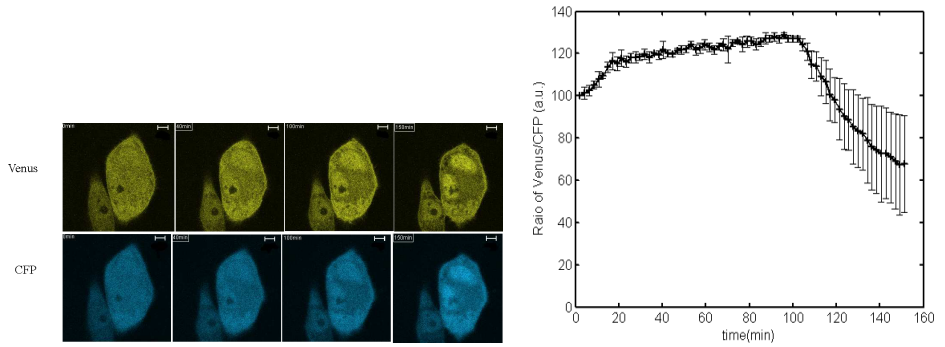


Fig. 3. XAP-induced morphological changes of the cells. Scale Bar: 10 μm.

nucleolus in healthy cells (Fig. 3). However, XAP induced cell swelling 3 h after XAP treatment and even induced disappearing of the nucleolus membrane 12 h after XAP treatment (Fig. 3).

3.4. *Dynamics of caspase-3 activation by XAP*

In order to the dynamics of caspase-3 activation induced by XAP, we used the dual channels time-lapse confocal fluorescence microscope to monitor the dynamical emission images of both the Venus and CFP channels. The cells chamber was placed in the temperature-control *37-2* before XAP treatment. The SCAT3 distributed evenly in the whole cell (Fig. 4A). However, XAP induced redistribution



(a) (b)

Fig. 4. Dynamics of SCAT3 distribution and caspase-3 activation induced by XAP. (A) Fluorescence image series of SCAT3 in single living cell after XAP treatment; (B) dynamics of caspase-3 activation induced by XAP. Scale Bar: 5 μm.

of the SACT3 (Fig. 4A), which gradually translocated to membrane and nuclear. Figure 4(B) shows the dynamics of Venus/CFP ratio of the recombinant FRET probe SCAT3 at the single-cell level every 5 min for a period of 3 h after XAP treatment. The emission ratio of Venus/VFP increased gradually until 100 min after XAP treatment, implied that XAP induced configuration change of the SCAT3; the emission ratio decreased rapidly 100 min after XAP treatment, implying that the SCAT3 was rapidly cleaved due to XAP treatment. This can be explained by the activation of caspase-3 in the stable transected ASTC-a-1 cells 2 h after the exposure to XAP. Cleavage of the SCAT3 resulted in decreased Venus/CFP ratio (Fig. 4B), indicating a decrease in FRET efficiency. Control experiments in ASTC-a-1 cells did not show a change in the YFP/CFP emission ratio and both the cyan and yellow channels (data not shown).

4. Discussions

The purpose of this investigation was to determine the effect of XAP on cell viability and caspase-3 activity. To this end, ASTC-a-1 cells were treated with XAP at a range of dose between 0 and 50 μl for 100 μl of DMEM. Cytotoxicity assay was then performed with Cell Counting Kit-8 after 48 h of XAP treatment. The results showed that high dose of XAP ($\geq 40\,\mu$l) caused a significant inhibition on the cells viability. At the same time, in order to determine the effect of XAP on caspase-3 activation, dynamic activation of caspase-3 was investigated during XAP-induced apoptosis by FRET technique. A FRET reporter SCAT3 was used to measure the dynamics of caspase-3 activity in living cells. Cells stably expressing SCAT3 reporter were imaged and analyzed after XAP treatment. Our results showed that caspase-3 was activated and then induced cell apoptosis. This study demonstrate that activation of caspase-3 does play an important role in XAP-induced cell apoptosis.

XAP may induce the redistribution of microtubule and microfilament, and then induce translocation of the YFP protein into membrane and nucleolus. The YFP translocation into cell nuclear may due to change of the viscosity in both the cytoplasm and nuclear. The mechanism of XAP-induced cell death needs to be further studied.

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References

1. H. Li and J. Yuan, "Deciphering the pathways of life and death," *Curr Opin Cell Biol* **11**, 261–266 (1999).
2. M. Rehm, H. Dussmann, R. U. Janicke, J. M. Tavare, D. Kogel and J. H. Prehn, "Single-cell fluorescence resonance energy transfer analysis demonstrates that caspase activation during apoptosis is a rapid process. Role of caspase-3," *J Biol Chem* **277**, 24506–24514 (2002).
3. Jiangsu New College of Medicine, "A dictionary of traditional Chinese drug," Shang-Hai, 1977, Shanghai Science and Technology Press, pp. 976.
4. G.-N. Li, "Yunnan Zhongyao Zhi," Kunming, 1987, Yunnan Science and Technology press, pp. 457.
5. K. Q. Luo, V. C. Yu, Y. Pu and D. C. Chang, "Measuring dynamics of caspase-8 activation in a single living HeLa cell during TNF-alpha-induced apoptosis," *Biochem Biophy Re Commu* **304**, 217–222 (2003).
6. R. B. Sekar and A. Periasamy, "Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations," *J Cell Biol.*, **160**, 629–633 (2003).
7. K. Takemoto, T. Nagai, A. Miyawaki and M. Miura, "Spatio-temporal activation of caspase revealed by indicator that is insensitive to environmental effects," *J Cell Biol.*, **160**, 235—243 (2003).
8. F. Wang, T. S. Chen, D. Xing, J. Wang and Y. X. Wu, "Measuring dynamics of caspase-3 activity in living cells using FRET technique during apoptosis induced by high fluence low power laser irradiation," *Lasers Surg Med*, **36**, 2–7 (2005).
9. Y. X. Wu, D. Xing, S. M. Luo, Y. H. Tang and Q. Chen, "Detection of caspase-3 activation in single cells by fluorescence resonance energy transfer during photodynamic therapy induced apoptosis," *Cancer Lett* **235**, 239–247 (2006).