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Methodlogical Report

Single Cell FRET Imaging for Determination of Pathway of Tumor Cell Apoptosis Induced by Photofrin-PDT

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KEY WORDS

apoptosis, caspase-3, caspase-8, fluorescence resonance energy transfer (FRET), SCAT3, photodynamic therapy (PDT), tumor necrosis factor-α (TNF-α)

ABBREVIATIONS

PDT	photodynamic therapy
ROS	reactive oxygen species
FRET	fluorescence resonance energy transfer
CFP	cyan fluorescence protein
YFP	yellow fluorescence protein
TNF-α	tumor necrosis factor-α

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ABSTRACT

Apoptosis is an important cellular event that plays a key role in pathogeny and therapy of many diseases. Apoptosis has been associated with photodynamic therapy (PDT) and its pathway is important in the mechanistic study of PDT. We show that single cell fluorescent imaging can be used to determine the pathway of PDT-induced tumor cell apoptosis. In this study, ASTC-a-1 tumor cells transfected by plasmid DNA SCAT3 were treated by Photofrin-PDT. The intracellular distribution of Photofrin was observed using a confocal microscope. The activations of caspase-3 and caspase-8 were dynamically observed using fluorescence resonance energy transfer (FRET). Our experimental results show that the Photofrin molecules are localized in cell mitochondria, and that after PDT caspase-3 was activated rapidly while caspase-8 remained inactive. These results demonstrate that the tumor cell apoptosis induced by Photofrin-PDT was directly initiated from the mitochondrial pathway.

INTRODUCTION

Photodynamic therapy (PDT) is a new cancer treatment modality which requires exposure of tumor cells to a photosensitizing drug followed by irradiation with light of appropriate wavelength.[1-3] The interaction between the excited photosensitizer and molecular oxygen produces singlet oxygen ($^1O_2$) as well as other reactive oxygen species (ROS) to induce cell death.[4-8] The intracellular localization of the sensitizer coincides with the primary site of photodamage.[9] PDT causes photooxidative damage to proteins and lipids that reside within a few nanometers of the photosensitizer binding sites.[4,6] Hence, the subcellular localization of a photosensitizer critically influences the kinetics and the regulatory pathways activated following PDT.[9] In response to PDT, apoptosis has been found to be a prominent form of cell death for many cell lines in tissue culture.[5]

Apoptosis is an important cellular event that plays a key role in pathogeny and therapy of many diseases.[10-11] It is believed to be associated with caspase activation and current evidence suggests two separable pathways leading to caspase activation. The extrinsic or death receptor pathway of caspase activation is initiated by the binding of a member of the tumor necrosis-factor (TNF)-family of death-receptor ligands to their cognate receptors (TNFR or Fas). Through their death domain, oligomerized receptors recruit adaptor protein (TRADD or FADD), which in turn recruits pro-caspase-8 and/or pro-caspase-10 to form the death inducing signaling complex (DISC). As a part of the DISC, pro-caspase-8 becomes autocatalytically activated and in turn cleaves effector pro-caspase-3/-7. The effector caspases then process different substrates, leading to apoptotic cell death.

The mitochondrial or intrinsic pathway of caspase activation involves the mitochondria as a central organelle. Different death signals, including PDT, can induce the permeabilization of the outer mitochondrial membrane, leading to the release of apoptogenic molecules such as cytochrome *c*, Smac/DIABLO and endonuclease G from the intermembrane space of the mitochondria. The release of these molecules is tightly controlled by different members of the Bcl-2 family. The release of cytochrome *c* promotes, in the presence of dATP/ATP, the formation of the apoptosome, which includes Apaf-1 (apoptotic protease activating factor-1) and pro-caspase-9. Autocatalytic activation of caspase-9 triggers the activation of effector caspases, ensuring apoptotic cell death.

Fluorescence resonance energy transfer (FRET) technique has been used widely to study protein-protein interaction in living cells.[12-14] Because the caspase-3 activation is a landmark event in apoptosis, assaying caspase-3 has been widely used as a tool for detecting programmed cell death.[15] SCAT3 was constructed as a FRET probe for the detection of

caspase-3 activation.[16] It consists of a donor (cyan fluorescent protein, CFP) and an acceptor (yellow fluorescent protein, YFP). The linking sequence contains a peptide DEVD.[17] The activation of caspase-3 leads to the cleavage of the linker, thus effectively reducing the FRET.[16]

Plasmid DNA Bid-CFP, in which CFP is fused to the C terminus of Bid, can also be used to study cell death mechanism through the activation of caspase-8, which leads to the cleavage of the Bid protein and yields two fragments, p15 and p7. An exposed glycine residue at the C terminus of Bid (p15) undergoes N-myristoylation, and the resulting CFP-p15 is translocated to the mitochondrial outer membrane. Therefore, the aggregation and localization of the reporter Bid-CFP to mitochondria represent the activation of caspase-8.[18] This noninvasive technique can help validate physiological events with high spatial and temporal resolution.[12-14] Using FRET technique based on SCAT3 and Bid-CFP, the spatio-temporal dynamics of caspase-3 and caspase-8 activation in individual living cells can be monitored in real-time.[16,18]

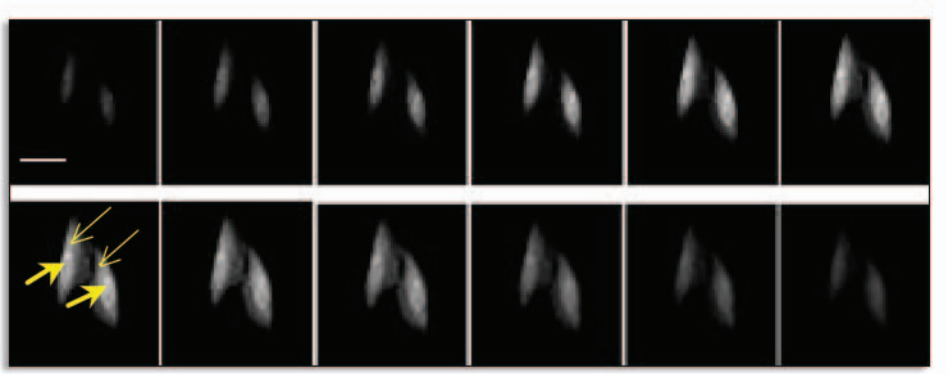

Figure 1. Transverse-imaging series of intracellular distribution of SCAT3 in two neighboring ASTC-a-1 cells using confocal microscopy. The scanning was performed from top to bottom of the cells (presented from left to right), with steps of 2 μm. Scale bar = 10 μm. The fluorescence emission is more intense in cell nucleus (thick arrows) than in cytoplasm (thin arrows).

In our previous studies, the SCAT3 has been used as the FRET probe during the laser irradiation of human lung adenocarcinoma (ASTC-a-1) cells.[19-20] Significant reduction of FRET, a strong indication of caspase-3 activation, was observed after treatment of PDT[19] and after high fluence low power laser irradiation.[20] However, the mechanisms of the initiation and regulation of PDT-induced apoptosis were not clear. In this study, confocal microscopy and SCAT3-based FRET were used to monitor the activation and the dynamics of caspase-3 and caspase-8 after PDT treatment, and to determine the pathway of PDT-induced tumor cell apoptosis.

MATERIALS AND METHODS

Cell culture. Human lung adenocarcinoma cell line (ASTC-a-1) was obtained from the Department of Medicine, Jinan University (Guangzhou, China), and cultured in RM1640 supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 μg/ml) in 5% CO_2 and 95% air at 37°C in a humidified incubator.

For the FRET experiments, the ASTC-a-1 cells were transfected by plasmid DNA SCAT3 (RIKEN Brain Science Institute, Japan) by incubating approximately $8x10^5$ ASTC-a-1 cells in a 25-ml flask with 200-μl serum-free medium containing 1 μg SCAT3 and 10 μl Lipofectin reagent (GIBCO/Invitrogen, Guangzhou, China) at 37°C for 8 hours. The cells stably expressing the SCAT3 reporters were screened with 1-ml 0.8 mg/ml G418 (GIBCO/Invitrogen). The positive clones were collected and cultured in complete medium for experiments.

The ASTC-a-1 cells were also transfected by plasmid DNA Bid-CFP (Gene Function Research Laboratory, Japan) by incubating approximately $8x10^5$ ASTC-a-1 cells in a 25-ml flask with 200-μl serum-free medium containing 1-μg Bid-CFP and 10-μl Lipofectin reagent (GIBCO/Invitrogen) at 37°C for 8 hours. Then the cells transfected with Bid-CFP reporter were incubated in complete medium. The transfected tumor cells were identified by their fluorescence emission.

PDT and tumor necrosis factor-α treatment of tumor cells. SCAT3-expressing ASTC-a-1 cells (0.8×10^5) were cultured on glass-bottomed 35-mm Petri dishes in 1-ml RM1640 supplemented with 10% FCS medium for 24 hours. To detect mitochondria-mediated apoptosis induced by PDT, cells were coincubated in the dark at 37°C with 10 μg/ml Photofrin (QLT Phototherapeutics, Vancouver, BC, Canada) for 20 hours before light irradiation. The light source was a He-Ne laser (HN-1000, Guangzhou, China; 632.8 nm). The cells were irradiated with a fluence of 10 J/cm^2 at a fluence rate of 10 mW/cm^2.

To detect the death receptor-mediated apoptosis, a separate batch of cells was exposed to 150 ng/ml TNF-α (Invitrogen, Guangzhou, China).

Confocal microscopy. To identify the spatial distribution of SCAT3 within the tumor cells, the fluorescence emissions by YFP (530 nm) from the cells were collected by a confocal microscope (MRC-600, BIO-RAD Laboratories, Hong Kong) with a 60x water-immersed objective lens and a 510-nm long-pass filter. To match the YFP excitation wavelength, a 10-mM Argon-ion laser with 488 nm light was used. The cells were scanned in the transversal direction with a 2-μm step.

To identify the subcellular localization of Photofrin, cells were incubated with Photofrin (20 μg/ml) in RM1640 medium at 37°C in the dark for 15 hours. To colocalize Photofrin, the cells were coloaded with 100 nM MitoTraker Red (Invitrogen), which localizes in cell mitochondria,[21] for 15 minutes at room temperature. A Laser Scanning Microscopes combination system (LSM510/ConfoCor2, Zeiss, Jena, Germany) with a 40x oil immersion planapochromat objective lens was used in this experiment. Confocal images of Photofrin fluorescence from the cells were obtained using a 458-nm excitation light from an argon laser and a 600-650 nm band pass barrier filter. Images of MitoTraker Red fluorescence from the cells were obtained using a 633-nm excitation light from the He-Ne laser and a 650-nm long-pass filter.

FRET Image acquisition and data analysis. To investigate the temporal profile of caspase-3 activation in single cells after PDT or TNF-α treatment, SCAT3-expressing ASTC-a-1 cells were treated with PDT or TNF-α as described above. During the treatment, the cellular fluorescence emission was collected with the LSM510 microscope. For excitation, the 458 nm line of an Ar-Ion laser was selected with an acousto-optical tunable filter, reflected by a dichroic mirror and focused through a Zeiss C-Apochromat 20x, NA 0.4 objective lens onto the cell sample. The emission fluorescence was split by a second dichroic mirror into two separate channels: the 470-500 nm band-filter (the CFP channel) and the 530 nm long-pass filter (the YFP channel). To quantify the results, the images of CFP and YFP emission were processed with Zeiss Rel3.2 image processing software (Zeiss, Germany). During He-Ne laser irradiation and imaging processes, cells were maintained at 37°C using a temperature regulator (Tempcontrol 37-2 digital, Zeiss). After FRET experiments, the ratio of the YFP/CFP emission was calculated.

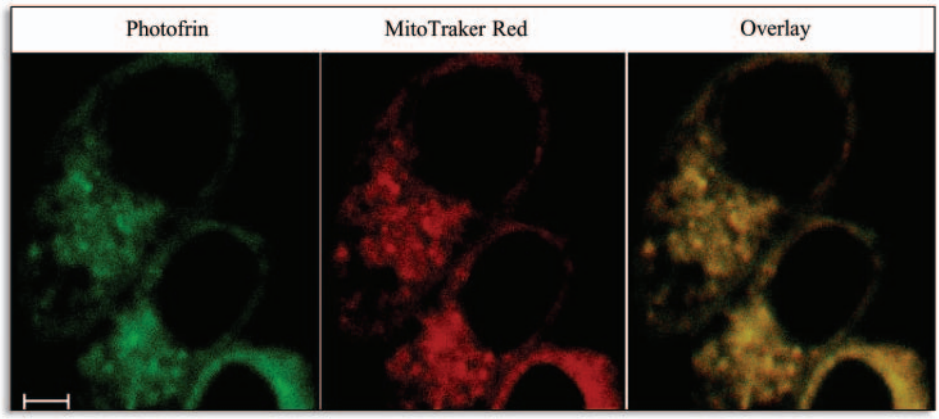

Figure 2. The fluorescence emission of ASTC-a-1 cells loaded with Photofrin (left panel), MitoTracker Red (middle panel) and their overlay image (right panel). The excitation wavelengths were 458 nm and 633 nm for Photofrin and MitoTracker Red, respectively. The collected emission wavelengths were 600–650 nm for Photofrin and >650 nm for MitoTracker, respectively. The matching fluorescence images of Photofrin emission and MitoTracker emission (the right panel) indicate that Photofrin is localized primarily in the mitochondria of the tumor cells. Scale bar = 5 μm.

RESULTS

Intracellular distribution and FRET efficiency of SCAT3. In order to detect the intracellular distribution of SCAT3 in the stably transfected ASTC-a-1 cells, the fluorescence emission of SCAT3 was collected by a LSM510 confocal microscope. YFP fluorescence was observed in both cytoplasm and nucleus, as shown in (Fig. 1). YFP fluorescence was more intense in the nucleus than in the cytoplasm, a result of higher expression of SCAT3 in nucleus.

To validate the FRET technique and to confirm the disruption of FRET in SCAT3-expressing ASTC-a-1 cells during apoptosis, the emission spectra of the cell suspension were obtained before and after the treatment of TNF-α. The fluorescence spectrum of untreated cells showed an emission peak around 530 nm, a clear indication of YFP dominated emission, while an emission peak around 480 nm appeared after the cells were treated, a clear indication of CFP dominated emission (data not shown).

Localization of photofrin in tumor cells. To determine the subcellular localization of Photofrin, fluorescence emissions from the cells were collected using the LSM confocal microscope after the ASTC-a-1 cells were loaded with the photosensitizer for 15 hours at 37°C in the dark. As shown in (Fig. 2) (left panel), Photofrin displayed a punctuated pattern of fluorescence emission primarily in the perinuclear area. To assess whether Photofrin binds to the mitochondria, emission spectra of cells coincubated with MitoTracker Red, a mitochondrion-specific dye, were also obtained (Fig. 2, middle panel). In the overlay emission image (Fig. 2, right panel), the Photofrin fluorescence corresponded closely to that of MitoTracker Red, indicating the mitochondrial localization of Photofrin in the tumor cells.

Rapid activation of caspase-3 in ASTC-a-1 cells during PDT-induced apoptosis. FRET was used to monitor the dynamics of caspase-3 activation in SCAT3-expressing ASTC-a-1 cells after PDT treatment. Figure 3 shows the fluorescence emissions of YFP and CFP from two neighboring cells. The intensity of YFP fluorescence decreased with time while that of CFP increased. The ratio of YFP to CFP fluorescence emission started decreasing about 20 minutes after PDT treatment, as shown in Figure 4. The decrease of the YFP/CFP ratio completed in less than 15 minutes, suggesting that the entire caspase-3 substrate was cleaved rapidly after PDT treatment.

Inactivation of caspase-8 in ASTC-a-1 cells during PDT-induced apoptosis. To investigate whether PDT-induced apoptosis in ASTC-a-1 cells could also be initiated by caspase-8 activation, Bid-CFP probes were used. To assess whether Bid-CFP aggregate and transfer to mitochondria, the cells transfected with Bid-CFP were also loaded with MitoTracker Red. Figure 5 shows typical temporal images of Bid-CFP emission and MitoTracker emission from a single cell after PDT treatment. After PDT, the Bid-CFP fluorescence was distributed in the entire cytoplasm (Fig. 5, upper panel). The fluorescence images of the MitoTracker (Fig. 5, lower panel) marked the location of mitochondria. There was neither aggregation nor colocalization of Bid-CFP in the mitochondria, as shown in (Fig. 5).

TNF-α-induced caspase-3 activation through death receptors-dependent pathway. The dynamics of caspase-3 activity was also studied using FRET during TNF-α induced apoptosis. Figure 6 shows typical time-course images of YFP and CFP emissions from several SCAT3-expressing cells after TNF-α treatment. The fluorescence intensity of YFP decreased while that of CFP increased about 180 minutes after the treatment. Figure 7 shows the temporal profile of YFP/CFP emission ratio, as measured in a single cell. After the administration of TNF-α (time 0), the YFP/CFP ratio remained unchanged for approximately 175 minutes and then decreased gradually. The ratio reached its minimum within 90 minutes.

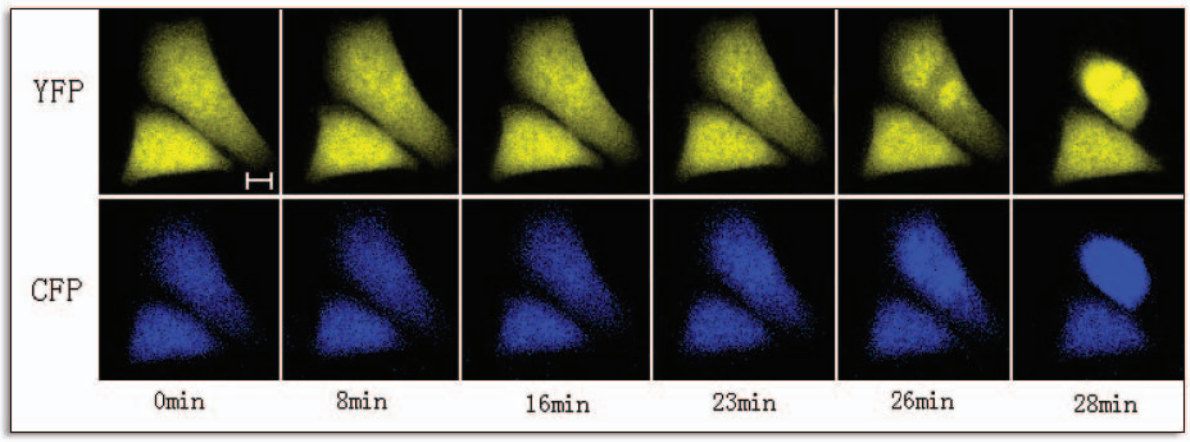

Figure 3. Fluorescence images of two neighboring SCAT3-expressing ASTC-a-1 cells after PDT treatment (10 J/cm^2 and 10 mW/cm^2, 632 nm). The images in the upper panel represent YFP emission while images in the lower panel represent CFP emission. The Scale bar = 5 μm.

DISCUSSION

Since the first report of apoptosis in PDT-treated cells,[5] it has been found to be a prominent form of cell death in response to PDT, evidenced by assays measuring either the fragmentation of DNA or the condensation of chromatin.[6-7,22-26]

Apoptosis can be induced by the ligation of plasma membrane death receptors, which stimulate the 'extrinsic' pathway, or by perturbation of intracellular homeostasis, the 'intrinsic' pathway.[27] Current evidence suggests that the most common pathway for apoptosis in PDT-treated cells involves mitochondria; however, other pathways, especially through caspase-8, may also be important, particularly when the dominant pathway is suppressed.[9] PDT inflicts damage to cells largely via ROS. The ROS travel only about several tens of nanometers before reacting with a molecule.[28] It is clear that the subcellular localization of photosensitizer coincides with primary site of photodamage and local photodamage to specific subcellular targets influences the kinetics and the regulatory pathways activated by PDT.[3,9,29-30]

It has been reported that procaspase-3 localizes in the cytoplasm and that caspase-3 activation is initiated in the cytosol. The activated caspase-3 redistributes to the nuclear compartment.[16,31] Plasmid DNA SCAT3, containing YFP and CFP linked by DEVD, was designed specifically to detect caspase-3 activation since the linker between YFP and CFP can be cleaved by activated caspase-3. The changes of fluorescence emission intensity between YFP and CFP are directly related to the caspase-3 activity.

In our current study, the SCAT3-transfected human lung adenocarcinoma cells (ASTC-a-1) were used. Figure 1 shows the distribution of SCAT3 in both cell cytoplasm and nucleus. The SCAT3-expressing tumor cells displayed the effect of FRET when treated by TNF-α. Before TNF-α treatment, two emission peaks at 480 nm (with a relatively low intensity) and 530 nm (with a relatively high intensity) were observed when excited with a 433-nm light. The strong 530-nm peak of CFP comes from photons transferred from CFP to YFP due to their linkage by DEVD. Eight hours after the TNF-α treatment, the peak at 530 nm disappeared and the peak of 480 nm was significantly enhanced (data not shown). This is the result of the SCAT3 DEVD sites being cleaved by caspase-3 and the FRET between YFP to CFP was disrupted.

Numerous reports have indicated mitochondria as important targets of PDT.[9] The binding of photosensitizers to mitochondria has been associated with the efficient induction of apoptosis.[32-34] In this study, we confirmed a strong mitochondrial localization of Photofrin in ASTC-a-1

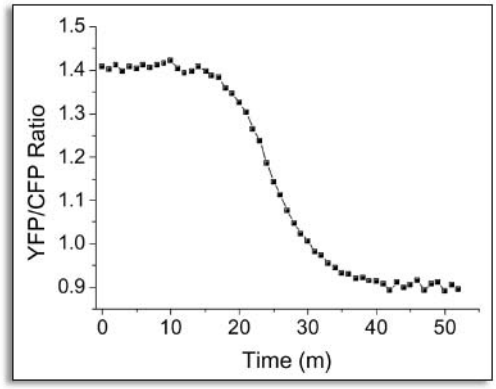

Figure 4. Temporal profiles of the YFP/CFP emission ratio for SCAT3-expressing ASTC-a-1 cells after PDT treatment. The ratio starts decreasing 20 minutes after PDT treatment and in 35 minutes the ratio reaches a stable level.

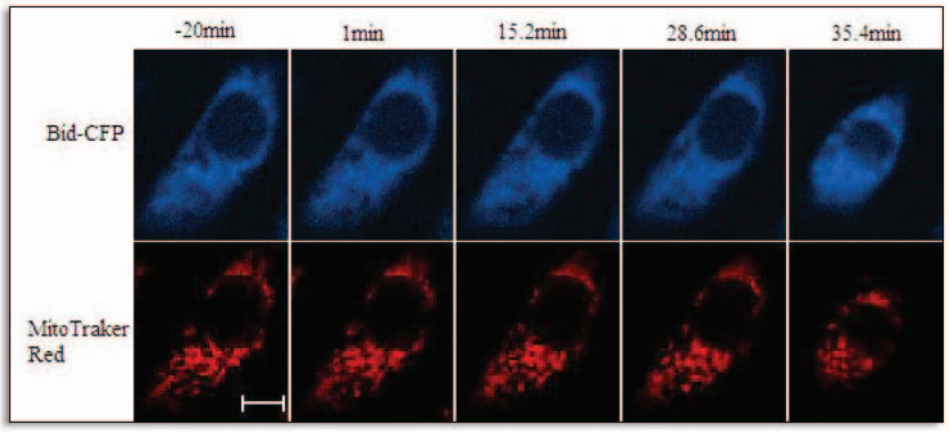

Figure 5. Images of ASTC-a-1 cells labeled with Bid-CFP and MitoTracker Red before (-20 minutes) and after PDT treatment (10 J/cm^2 and 10 mW/cm^2). The upper panel was obtained using the 458-nm excitation and the 470–490 nm light emission while the lower panel was obtained using 633-nm light excitation and >650 nm light emission. The uniform distribution of Bid-CFP in the entire cytoplasm after PDT (Upper panel) indicates that Bid-CFP is not cleaved, hence the lack of caspase-8 activation. Scale bar = 10 μm.

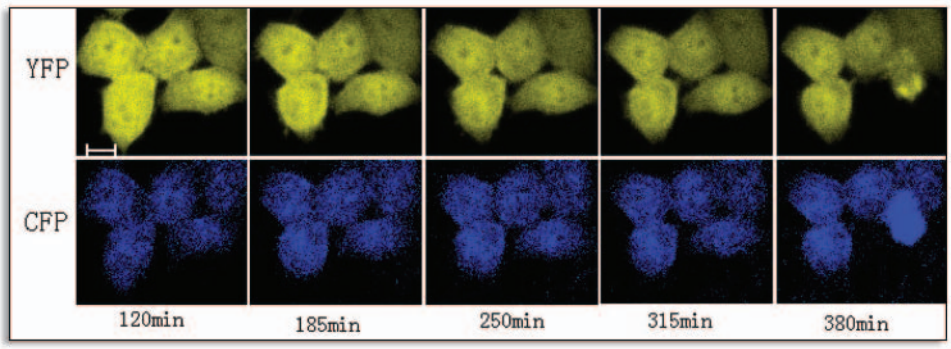

Figure 6. Image series of YFP and CFP in SCAT3-expressing ASTC-a-1 cells after TNF-α treatment (150 ng/ml). The intensities of emission remained unchanged during the first 120 minutes (data not shown). Noticeable changes in YFP and CFP fluorescence emissions start around 180 minutes after the treatment. Scale bar = 10 μm.

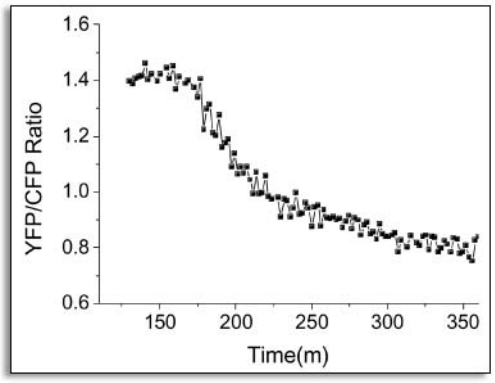

Figure 7. Temporal profile of the YFP/CFP emission ratio in SCAT3-expressing ASTC-a-1 cells after TNF-α treatment (150 ng/ml). The ratio remained unchanged during the first 180 minutes (data not shown). A noticeable decrease in the ratio started around 180 minutes and completed around 270 minutes after the treatment.

cells, as shown in Figure 2; the fluorescence emission of Photofrin corresponds spatially to the fluorescence emission of MitoTracker Red in the tumors.

Our experiments showed a rapid caspase-3 activation in the ASTC-a-1 cells after the Photofrin-PDT treatment, evidenced by the phenomena that the YFP emission decreased, while the CFP emission increased, shortly after PDT treatment, as shown by the tumor cell fluorescence images in Figure 3. The ratio of YFP/CFP emissions started a noticeable decrease about 20 minutes after the start of the PDT treatment and reached a stable level in 15 minutes, as shown in Figure 4, indicating a quick start and finish of caspase-3 activation by PDT treatment.

To study other possible mechanisms of PDT, such as the activation of caspase-8, we used Bid-CFP as a unique probe. The Bid-CFP aggregation to and localization in mitochondria represent activation of caspase-8.[18] Our experimental results showed a uniform distribution of Bid-CFP in the cell cytoplasma after PDT treatment (Fig. 5 upper panel). Clearly, PDT did not activate caspase-8; otherwise the fluorescence emission from Bid-CFP would have been concentrated in mitochondria, marked by the emission of MitoTracker Red (Fig. 5, lower panel). Therefore, it can be concluded that Photofrin-PDT induced apoptosis in ASTC-a-1 cells was initiated from the mitochondrial pathway.

TNF-α acts by binding to the TNFR receptors on the cell surface. Upon binding, TNFR associates with a platform adapter protein TRADD, which in turn recruits FADD to activate caspase-8.[35] Mature caspase-8 cleaves and activates effector caspase-3.[36] Using the SCAT3 transfected tumor cells and FRET technique, we studied the TNF-α induced cell death. As shown by the fluorescence images of tumor cells in Figure 6, the caspase-3 activation occurred 3 hours after the administration of TNF-α. This observation is comparable to the reported results of similar experiments using a different cell line.[16] The exhaustion of the SCAT3 probe is completed in 90 minutes, as shown by the time-course of YFP/CFP emission ratio in Figure 7, indicating that caspase-3 was activated slowly by upstream factors.

FRET, a noninvasive technique, can spatio-temporally monitor cellular events in different physiological conditions at a single cell level.[37-39] It has been utilized to study enzyme activity, protein location, protein translocation, small ligand binding, protein-protein interaction, conformational change, and posttranslational modification in real-time.[14] Specifically, FRET has been used to detect apoptotic signals that involve activation of different caspases,[15,17,40-41] interactions between Bcl-2 and Bax,[37-38,42-44] Ca^{2+} levels,[39,45-46] and other protein activities. In our current study, we employed single-cell FRET analyses to monitor the dynamics of caspase-3 and caspase-8 activation by PDT and TNF-α treatment. We clearly demonstrated the mitochondrial pathway of the Photofrin-PDT induced apoptosis. Furthermore, to our best knowledge, this is the first time that the temporal and spatial profiles of Photofrin-PDT induced cell apoptosis have been observed by FRET using SCAT3 reporters at single cell level. Our results demonstrate that FRET can be an effective method to precisely describe the initiation and dynamics of apoptosis and other mechanisms of cell death.

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