Measuring Dynamics of Caspase-3 Activity in Living Cells Using FRET Technique During Apoptosis Induced by High Fluence Low-Power Laser Irradiation

Fang Wang, MSc, Tong-Sheng Chen, PhD, Da Xing, PhD,* Jin-Jun Wang, MSc, and Yun-Xia Wu, MSc
Institute of Laser Life Science, South China Normal University, Guangzhou 510631, China

Background and Objectives: Low-power laser irradiation (LPLI) has been used for therapies such as curing spinal cord injury, healing wound etc. Yet, the mechanism of LPLI remains unclear. In order to determine the effects of high fluence LPLI on cell growth and caspase-3 activity, we have measured the dynamics of caspase-3 activity during cell apoptosis induced by high fluence LPLI treatment.

Study Design/Materials and Methods: He–Ne laser was used to irradiate human lung adenocarcinoma cells (ASTC-a-1). Cell Counting Kit-8 was used for cytotoxicity assay. A fluorescent microscope was used to perform fluorescence resonance energy transfer (FRET) imaging. A luminescence spectrometer was used to acquire the fluorescent emission spectrum. Statistical analysis was performed with Student’s paired t-test.

Results: Cytotoxicity assay showed that when light irradiation fluence exceeded 60 J/cm², LPLI treatment induced ASTC-a-1 cell apoptosis in a fluence-dependent manner. FRET imaging and spectrofluorometric analysis demonstrated that caspase-3 was activated during high fluence LPLI-induced cell apoptosis.

Conclusions: Using FRET technique, we have reported that high fluence LPLI can induce human lung adenocarcinoma cells (ASTC-a-1) apoptosis. The activation of caspase-3 plays an important role in the apoptotic process.

Key words: apoptosis; caspase-3; fluorescence resonance energy transfer (FRET); high fluence low-power laser irradiation (high fluence LPLI); living cells

INTRODUCTION

Low-power laser irradiation (LPLI) can modulate various biological processes [1]. LPLI can induce cell proliferation and differentiation [2,3]. It has been used to treat diseases of regeneration limitation [4] and to promote wound healing [5,6]; LPLI combined with photosensitizer is a form of photodynamic action and can induce cell apoptosis [7,8]; high fluence LPLI interferes with cell cycling and inhibits cell proliferation, thus, can be used to control certain types of hyperplasia [9–11]. Some reports suggest that, laser irradiation has a stimulatory effect on cell proliferation at relatively low fluences, but an inhibitory effect at high fluences [12,13]. Furthermore, the effect of LPLI also depends on laser wavelength, power density, irradiation procedure and the cell type [14,15].

Mechanism of LPLI remains unclear. There are literature reports show that, LPLI can induce expression or activation of certain signal proteins, such as extracellular signal-regulated kinase (ERK) [16], platelet-derived growth factor (PDGF) [18], vascular endothelial growth factor (VEGF) [19], nerve growth factor (NGF) [20], cyclinD1 [2], interleukin-8 (IL-8) [21], interleukin-1 alpha (IL-1α) [21]. LPLI can also induce changes in reactive oxygen species (ROS) production [22], Ca²⁺ concentration [23], ATP quantity [24], and mitochondrial membrane potential [24].

The fluorescence resonance energy transfer (FRET) technique has been used widely to study protein–protein interaction in living cells [25–27]. The principle of FRET has been previously described by Fields [25]. In summary, the emission spectrum of a donor fluorophore molecule at its excited state overlaps with the absorption spectrum of a proper acceptor fluorophore. When the two fluorophores are spatially close enough, there is energy transfer between the donor and acceptor molecules. The excited donor transfers its energy to the acceptor. This results in a reduction in donor fluorescence emission and, at the same time, an increase in acceptor fluorescence emission. Miura et al. [28] constructed a genetic FRET reporter, SCAT3. It consists of a donor cyan fluorescent protein (CFP) and an acceptor Venus (a mutant of yellow fluorescent protein). The donor and the acceptor are linked with a caspase-3 recognition and cleavage sequence (DEVD). The activation of caspase-3 leads to the cleavage of the linker, thus, effectively reduces the FRET. This noninvasive technique

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*Correspondence to: Dr. Da Xing, PhD, Institute of Laser Life Science, South China Normal University, Guangzhou 510631, China. E-mail: xingda@scnu.edu.cn

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can help validate the physiological events and can also provide multitude of spatio-temporal resolution [25–27]. Using FRET technique based on SCAT3, the spatio-temporal dynamics of caspase-3 activity in living cells can be monitored in real-time [28].

Apoptosis is a very important cellular event that plays a key role in pathogenicity and therapy of many diseases [29,30]. The mechanisms of the initiation and regulation of apoptosis are complex and diverse. Caspase family is closely connected with many apoptotic processes, while its member, caspase-3, being an important executive apoptotic molecule [31,32].

To investigate the cellular effect and mechanism of high fluence LPLI, human lung adenocarcinoma cells (ASTC-a-1) transfected with plasmid SCAT3 (pSCAT3) were irradiated and monitored noninvasively with both FRET spectroscopy and FRET imaging. The viability of the cells was evaluated 6 hours after the irradiation. The results show that, when light irradiation fluence exceeded 60 J/cm², LPLI significantly reduced the cell viability. With the FRET techniques, it was found that caspase-3 was activated and related to the subsequent apoptosis of the cells. We thus conclude that, given adequate time (thus fluence), low-power laser irradiation can induce caspase-3-related apoptosis.

MATERIALS AND METHODS

Cytotoxicity Assay After High Fluence LPLI

ASTC-a-1 cells were cultured in RPMI1640 supplemented with 10% FCS at a density of 5 × 10⁵ cells/well in 96-well microplate. Twenty-four hours after cultured, cells were divided into five groups and each group was irradiated by He–Ne laser (HN-1000, Guangzhou, China; 362.8 nm, 40 mW, spot diameter 0.635 cm) at fluence of 0, 15, 30, 60, and 120 J/cm², respectively. After irradiation, cells were cultured for 6 hours at 37°C, 5% CO₂. Cytotoxicity assay was then performed with Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). OD₄₅₀, the absorbance value at 450 nm, was read with a 96-well plate reader (DG5032, Hua dong, Nanjing, China), and the OD₄₅₀ is proportional inversely to the degree of cells apoptosis.

Cells Transfection and Screening of Cells Stably Expressing SCAT3 Reporter

Plasmid DNA of SCAT3 was transfected into ASTC-a-1 cells by using Lipofectin reagent (Invitrogen, USA). The cells stably expressing SCAT3 reporter were screened with 0.8 mg/ml G418, and positive clones were picked up with micropipettes. SCAT3 reporter was constructed by fusing of a CFP and a Venus with a specialized linker that contains a caspase-3 recognition and cleavage sequence (DEVD). Before caspase-3 is activated, CFP and Venus are covalently linked together, energy can be transferred directly from CFP to Venus, so fluorescence emitted from Venus can be detected when CFP is excited. Once caspase-3 is activated, the linker containing the DEVD will be cleaved, and CFP will separate from Venus, so the FRET effect of SCAT3 must decrease effectively [28]. pSCAT3 was presented by Dr. Miura.

Induction of Apoptosis by High Fluence LPLI and Living Cells Imaging Analysis

Cells stably expressing SCAT3 reporter were grown on a 35 mm culture dish. To measure the FRET effect in living cells, the culture dish was placed on the stage of a fluorescent microscope with 600 times magnification (Nikon Eclipse E600, Tokyo, Japan). Cells were irradiated by He–Ne laser (632.8 nm, 40 mW) at fluence of 60 J/cm² in the dark. During He–Ne laser irradiation and imaging process, cells were maintained at 37°C using a Thermo Plate (Tokhi Hit, Japan) in the whole experiment process. Before and after irradiation, temperature elevations of culture liquid were less than 0.2°C. After irradiation, cells were excited using an excitation filter (440–460 nm) plus a dichroic mirror of 455 nm. The emission images of CFP (460–490 nm) and Venus (520–560 nm) were recorded using a digital camera (Nikon, Tokyo, Japan) with 1,280 × 960 pixels resolution. Fluorescence intensities of CFP and Venus were obtained using the ImageTool software (UTHSCSA, USA).

Spectrofluorometric Analysis

Cells were grown on a 96-well plate. After 72 hours cultured, cells were irradiated by He–Ne laser (632.8 nm, 40 mW) at fluence of 60 J/cm² in the dark. After irradiation, cells were transferred into a quartz cuvette, which was then placed inside the sample holder of a luminescence spectrometer (LS55, Perkin Elmer, USA). The fluorescent emission spectra of 0 and 6 hours after high fluence LPLI were obtained by performing a spectrum scanning analysis of the luminescence spectrometer. The excitation wavelength was 433 nm, the excitation slit was 10 nm, the emission slit was 15 nm and the scanning speed was 200 nm/second. The corresponding background spectrum of culture liquid without cells must be subtracted.

Statistical Analysis

Data are represented as mean ± SEM. Statistical analysis was performed with Student’s paired t-test. Differences were considered statistically significant at P < 0.05.

RESULTS

Apoptosis of ASTC-a-1 Cells Induced by High Fluence LPLI

Cells were irradiated by He–Ne laser (632.8 nm, 40 mW) at fluence of 0, 15, 30, 60, and 120 J/cm², respectively. After irradiation, cells were cultured for 6 hours at 37°C, 5% CO₂. Cells apoptosis was analyzed using Cell Counting Kit-8. The OD₄₅₀ values for different irradiation fluence were showed in Figure 1. Compared with the control group, the cells irradiated at fluence of 60–120 J/cm² were significantly apoptotic (Student’s paired t-test, P < 0.001), but the cells irradiated at fluence of 15–30 J/cm² were almost not apoptotic.

FRET Effect of SCAT3 for the Control Was Unchanged With Time

SCAT3 reporter can be used to provide a quantitative measurement of the caspase-3 activity in living cells. The
fluorescence intensity ratio between Venus and CFP emission was related to the caspase-3 activity. The ratio can be determined through the pixel value of the Venus and the CFP image. As a reporter for caspase-3 activity, it is important that the FRET effect of SCAT3 is sensitive only to caspase-3 activation. Figure 2A showed a typical time-course of fluorescent images of Venus and CFP from two neighboring cells stably expressing SCAT3 reporter. From the images, fluorescence intensities of Venus and CFP were nearly unchanged. This demonstrated that FRET effect was unchanged and caspase-3 was not activated. The average emission ratio of Venus/CFP was showed in Figure 3 and it was plotted at different times from five different cells. The ratio was almost constant. These results demonstrated that the reporter was not cleaved and was resistant to environment. Based on the results, SCAT3 is a wonderful reporter for detection of the caspase-3 activity.

**Dynamics of Caspase-3 Activity in High Fluence LPLI-Induced Cell Apoptosis**

We have used FRET imaging method to determine the dynamics of caspase-3 activity during high fluence irradiation (60 J/cm²)-induced cell apoptosis. In order to eliminate the energy deposition of high fluence LPLI, the culture dish was placed on a Thermo Plate installed on the stage of the fluorescent microscope. By this way, cells were maintained at 37 °C in all of our experiments. Temperature elevations of culture liquid were less than 0.2 °C before and after irradiation by He–Ne laser (632.8 nm, 40 mW) at fluence of 60 J/cm². The typical time-course images of Venus and CFP from six neighboring cells stably expressing SCAT3 reporter were showed in Figure 2B. From these images, fluorescence intensities of Venus decreased gradually, while that of CFP increased gradually. The average emission ratio of Venus/CFP was plotted after different times of high fluence LPLI treatment from five different cells (Fig. 3). These results demonstrated that the caspase-3 activity increased gradually with time after high fluence LPLI treatment. The average time of the onset of caspase-3 activation was about 60 minutes (n = 5) after high fluence LPLI treatment. The caspase-3 activation had increased gradually for about 200 minutes (n = 5). At about 260 minutes after high fluence LPLI, cell apoptosis occurred obviously from cells shrinkage and nuclei condensation (Fig. 2B), and it is evident that caspase-3 was activated.
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preceding cell apoptosis. These results showed that high fluence LPLI can activate caspase-3, and then induce cell apoptosis.

Spectrofluorometric Analysis of Caspase-3 Activity During High Fluence LPLI-Induced Cell Apoptosis

In order to further demonstrate caspase-3 activity during high fluence LPLI-induced cell apoptosis, we use a spectrometer to measure the change of FRET effect in response to high fluence LPLI treatment. The results were showed in Figure 4, the bold line was the spectrum obtained after 6 hours of high fluence LPLI treatment, and the other for the control. When cells stably expressing SCAT3 reporter were excited at excitation wavelength for CFP (433 nm), most fluorescence was emitted from Venus (527 nm) and emission peak at 476 nm (CFP) was low for the control, which indicated that there was a very strong FRET effect and caspase-3 was not activated. Yet, contrast to the control, after 6 hours of high fluence LPLI treatment, the emission peak at 527 nm decreased and the peak at 476 nm increased. This result indicated that FRET effect decreased largely and the caspase-3 activity increased. These results further verified that caspase-3 was activated during high fluence LPLI-induced cell apoptosis.

DISCUSSION

It was the purpose of this investigation to determine the effect of high fluence LPLI on cell growth and caspase-3 activity. To this end, ASTC-a-1 cells were irradiated (632.8 nm, 40 mW) at a range of fluence between 0 and 120 J/cm². Cytotoxicity assay was then performed with Cell Counting Kit-8 after 6 hours of high fluence LPLI treatment. The results showed that high fluence LPLI (>60 J/cm²) caused a significant apoptosis. At the same time, in order to determine the effect of high fluence LPLI on caspase-3 activity, dynamic activation of caspase-3 was investigated during high fluence LPLI-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 high fluence LPLI (60 J/cm²) treatment. Results showed that caspase-3 was activated and then induced cell apoptosis. This study demonstrated that activation of caspase-3 does play an important role in high fluence LPLI-induced cell apoptosis.

ASTC-a-1 cells irradiated at fluence >60 J/cm² were significantly apoptotic. This inhibitory effect on cell culture at high fluence is consistent with the studies reported by O’Kane et al. [9], who found a decrease in thymidine incorporation after irradiation with He–Ne laser (660 nm) at dose >5.8 J/cm² on haemopoietic cell lines. Gross and Jelkmann [10] also found growth inhibition after repeated irradiation with He–Ne laser (632.8 nm, 40 mW/cm²) at dose >11.9 J/cm² on kidney epithelial cells, and Lubart et al. [12] reported that the number cells in mitosis decreases after irradiation with He–Ne laser (630 nm, 2 mW/cm²) at dose >60 J/cm² on fibroblast cell line. All reports about the irradiation of low-power laser to cell culture in vitro demonstrate that biostimulation depends on energy density, irradiation procedures and cell type, the last probably being the most important factor.

FRET, a noninvasive technique, can spatio-temporally monitor cell events in physiological condition in single living cell [25–27]. It has been utilized to reveal the dynamic activity of enzyme, and it provides a view of protein location, protein translocation, small ligand binding, protein–protein interaction, conformational change, and posttranslational modification in real-time [27]. This can’t be fully elucidated by traditional biophysical or biochemical approaches, which can only measure the average behavior of cell populations and the static spatial information available from fixed cells and thus can’t provide direct access to cells life event in their natural environment [27]. So we use FRET technique to dynamically measure activity of caspase-3 in living cells.

High fluence LPLI induced cell apoptosis by activating caspase-3. High fluence LPLI-induced DNA damage has been detected using the single cell Comet assay [33]. Caspase family is nearly connected with many apoptotic processes. Caspase-3, cutting DNA molecule and inducing DNA damage, is an important executive apoptosis molecule [31,32]. Aside, during PDT-induced cell apoptosis, apoptosis was mediated by sequential activation of two caspase cascades, the first is caspases 8, 3, and 6, and the second is caspases 9, 3, and 6 [34]. By FRET, we found that the caspase-3 activity in living ASTC-a-1 cells increased gradually after high fluence LPLI treatment. Our results were contributed to clarity of mechanism of inhibitory effect of high fluence LPLI.

![Fig. 4. Spectrofluorometric analysis of caspase-3 activation.](image-url)
It is also possible that high fluence LPLI induces caspase-3 activation and cell apoptosis by internal photodynamic action. Lubart et al. [35] postulated that high fluence LPLI produces large amounts of singlet oxygen. The singlet oxygen oxidizes rapidly a large variety of biological molecules and damage DNA, and these events are responsible for cell apoptosis. This postulation is conceivable, because photodynamic therapy (PDT) can induce cell apoptosis [36,37] and there are a few of endogenous photosensitizer in vivo such as α-aminolevulinic acid (α-ALA) [38], protoporphyrin IX [39], riboflavin (RF) [40], tryptophan (Trp) [41] and so on. When photosensitizers are exposed to a specific wavelength of light such as He–Ne laser, they produce a form of oxygen that kills nearby cells [36,37].

In conclusion, this study proved that human lung adenocarcinoma cells (A549-a-1) apoptosis can be induced by high fluence LPLI (>60 J/cm²) and the activation of caspase-3 was involved in it.

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REFERENCES


