Original Research

Label-free detection for radiation-induced apoptosis in glioblastoma cells

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Abstract

Current flow cytometry (FCM) requires fluorescent dyes labeling cells which make the procedure costly and time consuming. This manuscript reports a feasibility study of detecting the cell apoptosis with a label-free method in glioblastoma cells. A human glioma cell line M059K was exposed to 8 Gy dose of radiation, which enables the cells to undergo radiation-induced apoptosis. The rates of apoptosis were studied at different time points post-irradiation with two different methods: FCM in combination with Annexin V-FITC/PI staining and a newly developed technique named polarization diffraction imaging flow cytometry. Totally 1000 diffraction images were acquired for each sample and the gray level co-occurrence matrix (GLCM) algorithm was used in morphological characterization of the apoptotic cells. Among the feature parameters extracted from each image pair, we found that the two GLCM parameters of angular second moment (ASM) and sum entropy (SumEnt) exhibit high sensitivities and consistencies as the apoptotic rates (P_a) measured with FCM method. In addition, no significant difference exists between P_a and ASM_S, P_a and SumEnt_S, respectively ($P > 0.05$). These results demonstrated that the new label-free method can detect cell apoptosis effectively. Cells can be directly used in the subsequent biochemical experiments as the structure and function of cells and biomolecules are well-preserved with this new method.

Keywords: Glioblastoma cell, apoptosis, cell morphology, automatic feature extraction, polarization diffraction imaging, flow cytometry

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Introduction

Glioblastomas and malignant gliomas are considered to be the most common primary brain tumors which are closely related to poor quality of life and the dismal prognosis.[1] Clinical studies have shown that radiation therapy combined with chemotherapy is the most effective adjuvant treatment modality for virtually all patients with high-grade glioma.[2] Investigation of mechanism of how the biological behavior of tumor cells is affected by radiation has attracted a lot of research interests in recent years.[3]

Programmed cell death will occur in cells with severe DNA damage induced by ionizing radiation.[4] DNA damage induced by radiation can arouse a sequence of intracellular biochemical reactions and ultimately leads to apoptosis.[5] Many genes and their products are involved in these processes, and the regulatory mechanisms are very complex.[6] The recognition of apoptotic cell is increasingly important in study of genes and proteins involved in DNA repair and cell apoptosis.

In recent years, due to its throughput, flow cytometry (FCM) has become the most widely used method for quantitative detection of cell apoptosis. One of commonly used protocols for FCM assay involves cell staining with two fluorescent reagents, Annexin-FITC and propidium iodide (PI) followed by flow measurement.[7] Annexin-FITC can specifically recognize phosphatidylserine (PS) exposed to extracellular leaflet of the plasma membrane in the early stage of apoptosis. In addition, PI is permeant to apoptotic cells and combines with nucleic acids in the late stage of apoptosis.[8] Hence, early and late apoptotic cells can be identified through accompanied with the two reagents.[9] The distribution of viable cells, early and late apoptotic cells is completely different in the forward scatters (FSC)/side scatters (SSC) plots obtained with FCM assay.[10,11] Therefore, the morphological

changes in apoptotic cells can be correlated with the feature of their scattered lights.[9]

FCM assay is the most widely used method now which requires fluorescent dyes for cell labeling. This makes the experimental procedure costly and time consuming. And the cells labeled with fluorescent dyes cannot be directly used in biological experiments. Parallel experiments are necessary to study the genes and proteins involved in apoptosis, which means more cells are needed and the procedure and cost of the experiment are increased. It is worth emphasizing that the primary cells are difficult to obtain and the proliferation could be very slow. It has been reported that gene expression of primary cells may change after repeated amplification and subculture.[12] Therefore, an efficient and label-free method for recognition and quantification of cell apoptosis is still highly demanded.

In this feasibility study, a new label-free method was explored to detect cell apoptosis without fluorescent staining. M059K cells were separated into two groups after irradiation. One was used to benchmark the apoptotic rate with FCM assay and the other was used in the measurement with the newly developed label-free method named polarization diffraction imaging flow cytometer (p-DIFC) to investigate cellular structure changes indirectly through feature analysis of the scattering images.[13–15] A gray level co-occurrence matrix (GLCM) algorithm was used for the extraction and analysis of characteristic parameters.

Materials and methods

Experimental design

Human malignant glioma cell line M059K was rendered apoptosis using irradiation at dose of 8 Gy.[16] Cells were collected into 15 mL centrifuge tubes and span at 1000 rpm for 3 min at different time points (tps) after the radiation treatment. Detection of apoptotic rate (P_a) in M059K cells at each tp was conducted with two methods: (1) FCM assay with Annexin V-FITC and PI and (2) label-free method of p-DIFC with feature quantification of diffraction images. All the experiments were repeated three times and statistical analysis was performed. We calculated the values of coefficient of determination (R^2) on the tps dependence between the two methods. Correlations are defined as excellent for $R^2 \geq 0.90$, very good for $0.90 < R^2 \leq 0.75$, good for $0.75 < R^2 \leq 0.50$, moderate for $0.50 < R^2 \leq 0.25$, and poor for $R^2 < 0.25$.[17]

Cell culture

The M059K cells were incubated in DMEM/F12 (1:1) medium supplemented with concentrations of 10% fetal bovine serum, 1 mmol/L L-glutamine, 100 I.E/mL penicillin, 100 g/mL streptomycin, and 2.5 mg/mL fungizone. The cell culture environment was set up at 37°C, 5% CO_2, and 80% humidity.

Irradiation

Cells were cultured in 6-cm culture dishes until they reached 80% confluent and were irradiated with the dose of 8 Gy from a photon beam of 6 MV with a clinical linear accelerator (Clinac 600CD, Varian Medical Systems). Apoptosis of the cells were then analyzed at tps of 0 h, 1 h, 7 h, 24 h, 31 h, and 48 h after irradiation. Meanwhile, untreated cells were used as blank control. That is, each group of cells included one control cell sample and five treated cell samples.

Apoptosis measurement with FCM assay

Cell concentration was adjusted to $3–6 \times 10^5$ cells/mL for the measurements. The apoptosis rates at different tps post-irradiation were measured with FCM assay as the reference. The measurements for both the control and treated cell samples were conducted at different tps utilizing Annexin V-FITC, PI, and the Accuri C6 Flow Cytometer (BD Biosciences) with the standard Annexin V-FITC/PI assay protocol for FCM assay.[18,19] The apoptosis rates at each tp were recorded.

Apoptosis measurement with label-free method

Another group of cell samples was then quantified with the label-free method and an in-house p-DIFC system. Diffraction images record angle-resolved distribution of scattered light from a particle excited by coherent light and can correlate highly with the 3D morphology of a particle.[20] Cell apoptosis is accompanied by changes in cell morphology. So through the analysis of the texture in diffraction images, we can obtain the information on structural changes of cells undergoing apoptosis.[20,21]

After M059K cells were collected and span at 1000 rpm for 3 min at different tps, they were prepared in DMEM/F12 (1:1) media with concentration of $1.5–3 \times 10^5$ cells/mL and volume of 1 mL. 1000 pairs of raw diffraction images at different tps were acquired after pushing the cell suspension of each sample into the system's core fluid channel. The image files were processed and analyzed with an in-house software based on GLCM algorithm which had been applied for characteristic parameters extraction of image texture and intensity.[13] The GLCM is a matrix consisting of joint probabilities of intensity or gray level between the two adjacent pixels in the image.[13] In this study, 38 image parameters were extracted from each pair of diffraction images to investigate their dependence on different tps.

Statistical analysis

The mean values and standard deviations of P_a and GLCM parameters were calculated to evaluate the statistical significance between the two measured results. R^2 values on the tps were also calculated in order to evaluate the correlation of the apoptotic rate P_a measured with FCM assay and the two GLCM parameters measured with the p-DIFC method. SigmaPlot (12.0, Systat Software) was used in the analysis.

Results

FCM assay

The representative data of control and irradiated cell samples measured with FCM assay at different tps are shown in Figure 1. Figure 1(a) is from the control cell sample. Figure 1(b–f) is from the treated cell samples measured at tps of 1 h, 7 h, 24 h, 31 h, and 48 h after irradiation, respectively. The lines in the figure were placed by an experienced researcher and the apoptotic rate P_a was calculated accordingly. Out of all four, the lower left quadrant displays normal cells (FITC-, PI-), the upper right quadrant (R1) shows the late apoptotic and necrotic cells (FITC+, PI+), and the lower right quadrant (R2) represents the early apoptosis cells (FITC+, PI−).[22] The figure shows the measurement result of 10,000 M059k cells. Although the FCM assay can detect apoptosis rapidly, uncertainty exists in determining P_a since the lines are placed subjectively rather than objectively. Figure 1 shows that apoptotic rates increase along with the post-radiation time, and the result provides a basis for the subsequent measurement.

p-DIFC measurement

The scattered light in p-DIFC were divided into two cross-polarized (s-polarized and p-polarized) components by a polarizing beam splitter. A pair of diffraction images obtained with p-DIFC from a single cell consists of two images, one from s polarization and the other from p polarization. Process and analysis of the raw images with the GLCM-based software generated 38 parameters, 19 on each of the s and p directions. Figure 2 presents the typical diffraction image pairs.

Among the 38 GLCM parameters, two were sensitive to tps with smaller fluctuations between cell samples which were shown in Figure 3(a and b). The curve in Figure 3(c) shows the mean values of P_a measured with FCM assay. The error bars in the figure show the standard deviations of P_a. The results in Figure 3 indicate that the parameter of ASM_s increases with tps while SumEnt_s decreases. Angular second moment (ASM) measures the homogeneity of the images.[23] A homogeneous pattern with a few gray levels will give a GLCM that has a few elements with relatively high values, i.e. when an image is homogenous, the value of ASM will be high which indicates textural uniformity. Similar to entropy, the parameter of Sum Entropy (SumEnt) measures the irregularity and randomness among gray levels in the diffraction images. In order to compare the relative relationship between the data and find how the parameters changed over time, the ASM and SumEnt values at corresponding tps were normalized to each sum value of the six tps, respectively. The results shown in Figure 3 indicate that p-DIFC assay is capable of quantifying morphological changes and detecting apoptosis of individual cells.

FCM vs. p-DIFC

Apoptotic rates obtained with FCM assay and the two parameters of ASM_s and SumEnt_s obtained with the new

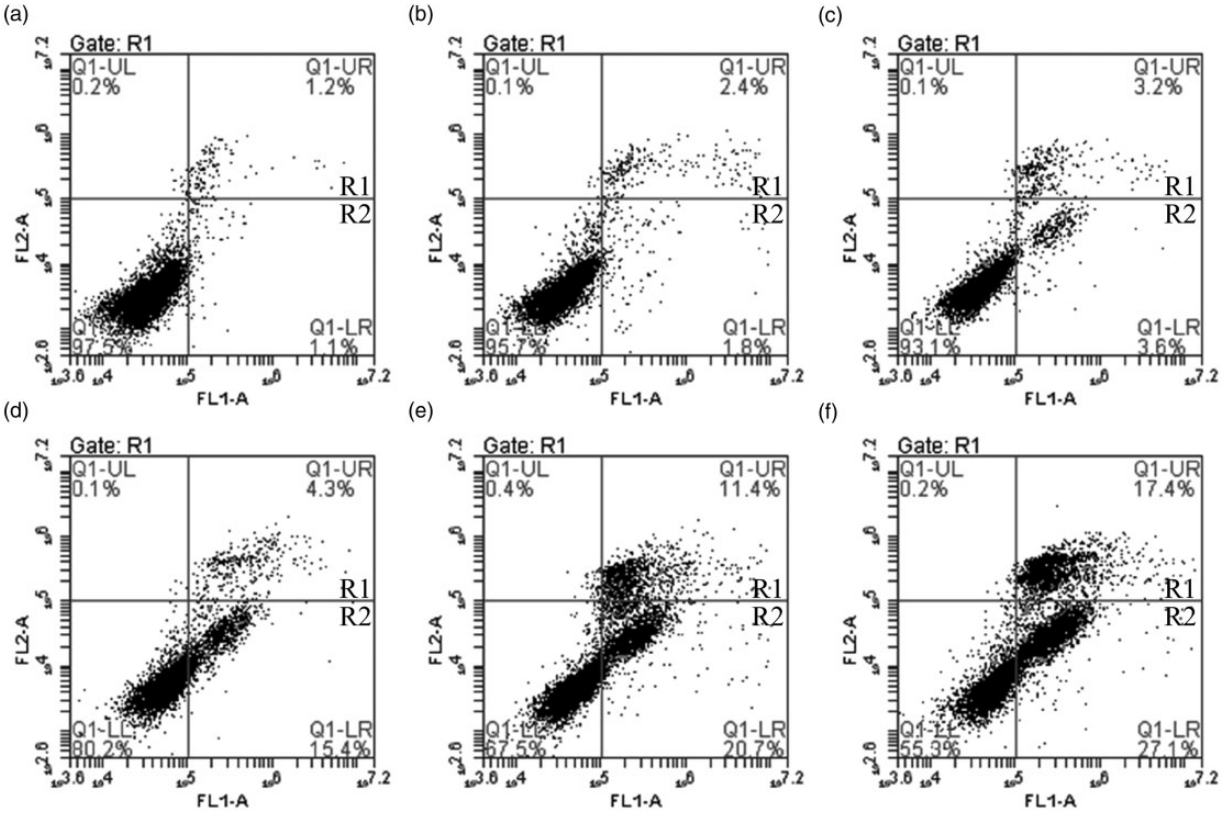

Figure 1 Scatter plots of M059k cells after 8 Gy irradiation at different time intervals acquired in the Annexin V-FITC/PI assay. (a) From control cell sample; (b)–(f) from irradiated treated cell samples at tps of 1 h, 7 h, 24 h, 31 h, and 48 h

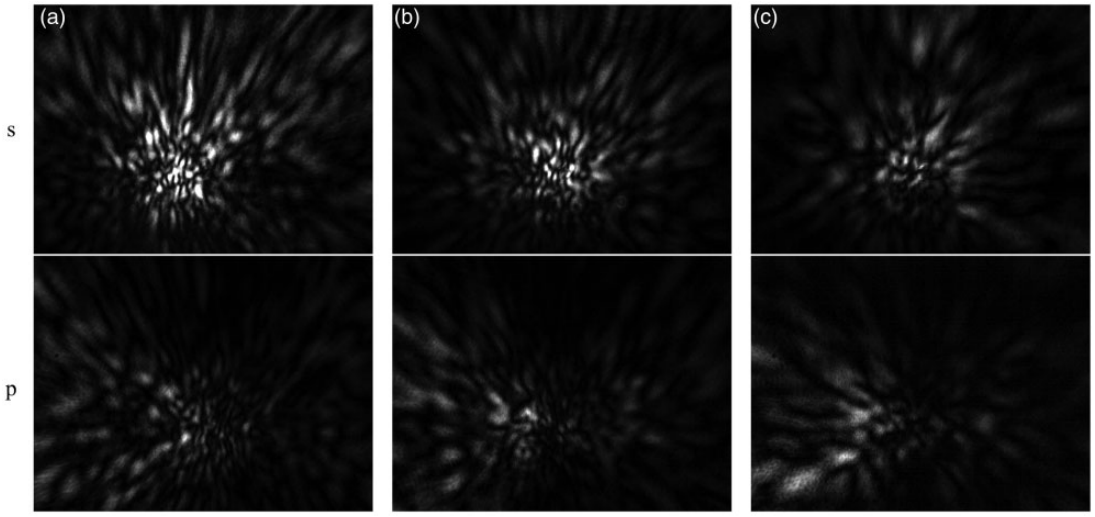

Figure 2 Examples of diffraction image pairs acquired from individual flowing cells at different time points. (a) $t = 0$ h; (b) $t = 24$ h; (c) $t = 48$ h. Letter s and letter p represent s-polarization and p-polarization of the scattered light

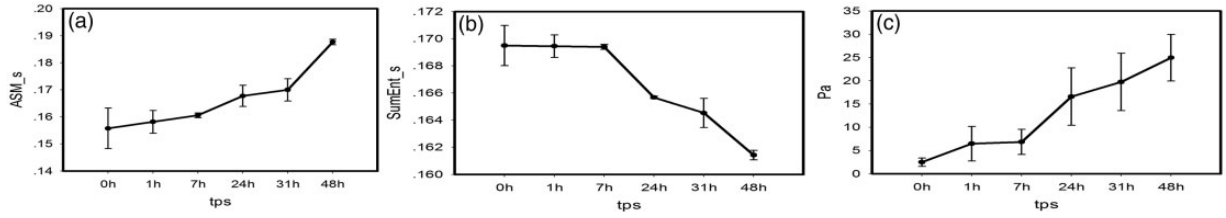

Figure 3 The parameters measured at tps of 0 h, 1 h, 7 h, 24 h, 31 h, and 48 h after irradiation. (a) The parameter of ASM_s obtained with p-DIFC; (b) the parameter of SumEnt_s obtained with p-DIFC; (c) the mean values of P_a measured with FCM assay. The error bars show the standard deviations

Table 1 Apoptotic rates (P_a) measured with FCM assay and the GLCM parameters (ASM_S and SumEnt_S)

		Parameters		One-way ANOVA test	
Time	**P_a (%)**	**ASM_S**	**SumEnt_S**	**P_a–ASM_S**	**P_a–SumEnt_S**
0 h	2.51 ± 0.90	0.155 ± 0.007	0.1695 ± 0.001	$P = 0.300$	$P = 0.303$
1 h	4.28 ± 3.71	0.158 ± 0.004	0.1694 ± 0.0008	$P = 0.820$	$P = 0.661$
7 h	6.84 ± 2.69	0.161 ± 0.0009	0.1693 ± 0.0002	$P = 0.407$	$P = 0.326$
24 h	19.74 ± 6.17	0.168 ± 0.004	0.1657 ± 0.0001	$P = 0.184$	$P = 0.209$
31 h	30.51 ± 6.16	0.170 ± 0.004	0.1645 ± 0.001	$P = 0.066$	$P = 0.051$
48 h	38.45 ± 7.85	0.188 ± 0.001	0.1614 ± 0.0003	$P = 0.068$	$P = 0.053$

method at each tps are listed in Table 1. These data show that no significant difference exists between P_a and ASM_S, P_a and SumEnt_S, respectively ($P > 0.05$). Calculation of R^2 on the tps between the two GLCM parameters and P_a is summarized in Table 2. It shows that ASM_s and SumEnt_s exhibit good correlations with P_a and demonstrates that the label-free detection using DIFC can reflect the process of apoptosis by analyzing the structural changes in the cells. Since the diffraction image texture directly depends on the shape and structure of cells and apoptosis is accompanied by a series of changes in cell morphology, it is considered that changes in ASM_s and SumEnt_s are decided by the sum of the three-dimensional structure changes in the increasing apoptosis as time increasing. Therefore, ASM_s and SumEnt_s are indicative of cell apoptotic status.

Discussion

Quantitative detection of cell apoptosis plays an important role for studying the molecular mechanism of DNA damage and repair, and death progression of cells. FCM has the advantages of rapidness, efficiency, and high sensitivity, and has been widely used for the investigation of cell

Table 2 The values of R^2 on the correlation between the dependences of GLCM parameters and P_a by FCM on tps

	ASM_s	SumEnt_s
P_a by FCM	0.940	0.980

apoptosis. However, the conventional FCM assay needs to stain the cells and the same sample cannot be directly used in subsequent biological experiments. Different from the FCM assay, the new label-free method can obtain diffraction images of cells through the measurement of light scattering signals which reflect the internal refractive index distribution of the cells.[24,25] Previous study of light scattering by single cells has shown the correlation between the spatial distribution of scattered light intensity and the morphology of cells.[13] According to reported studies, a variety of biochemical events which are remarkably similar across cell types and species cause characteristic morphologic changes of apoptotic cells,[26] such as membrane blebbing, cell shrinkage, fragmentation of nuclear, and chromatin condensation which would lead to changes of intracellular refractive index distribution that can be identified by our new method and generates diffraction images with significant difference at the different tps after irradiation. Observable differences in the patterns of diffraction images of the normal and apoptotic cells have been revealed in our study as shown in Figure 2. Analysis has shown that the two GLCM parameters, ASM and SumEnt, exhibit sensitive dependence on tps as presented in Figure 3 and could be used as the morphological feature parameters for detection of apoptosis.

Compared with conventional methods, p-DIFC is a rapid and cost-effective approach. The p-DIFC method can reduce the experimental procedures efficiently due to the elimination of cell staining process which extremely shortens the time from sample collection to obtaining measurement results. These advantages are more prominent when dealing with a large number of samples. A valuable application potential exists in the field of clinical medicine and basic research since the new technique can preserve function of the genes and proteins while measuring cell structure information. Cell samples measured can be directly used in studies of the proteins, the mutual regulations between them, and the interaction between the genes and proteins. However, experimental study with clinical tumor samples is still very challenging as heterogeneity is a typical feature of tumor samples and existing techniques for separation of the cancer cells in a sample are all label based. The p-DIFC hardware system is being improved in the effort of moving this technique to investigation with clinical tumor samples.

In conclusion, we have reported a label-free detection of radiation-induced apoptosis in glioblastoma cells with a p-DIFC method without staining. The new technology may have unique advantage in detection and quantification of the cell apoptosis in cerebrospinal fluid of patients with glioma and ascites of patients with ovarian cancer for evaluation of treatment response.

Author contributions: YS and YF designed the study; DQ, CY, CJ, and YS conducted the experiments; DQ, JF, and YF conducted the data analysis and interpretation of the studies; DQ, JF, and YF drafted the manuscript; all authors participated in the revision and review of the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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