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Short communication

Application of microfluidic gradient chip in the analysis of lung cancer chemotherapy resistance

Wang Siyan[a,c], Yue Feng[b], Zhang Lichuan[c], Wang Jiarui[c], Wang Yingyan[d], Jiang Li[c], Lin Bingcheng[e], Wang Qi[f,*]

[a] Department of Emergencyt, The Second Hospital Affiliated to Dalian Medical University, 467 Zhongshan Road, Dalian 116023, China
[b] Department of Urology, Dalian Municipal Friendship Hospital, 47 Renmin Road, Dalian 116001, China
[c] Graduate School of Dalian Medical University, 9 West Part, Lvshun Southern Road, Dalian 116044, China
[d] Diagnostics Laboratory Center, Dalian Medical University, 9 West Part, Lvshun Southern Road, Dalian 116044, China
[e] Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, China
[f] Department of Respiratory Medicine, The Second Hospital Affiliated to Dalian Medical University, 467 Zhongshan Road, Dalian 116023, China

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ABSTRACT

The major challenge of chemotherapy is the disease resistance for many lung cancer patients. Integrated microfluidic systems offer many desirable characteristics and can be used in cellular biological analysis. This work aimed to study the correlation between the expression of Glucose Regulated Protein-78 (GRP78) and the resistance to anticancer drug VP-16 in human lung squamous carcinoma cell line SK-MES-1 using an integrated microfluidic gradient chip device. We used A23187, a GRP78 inducer, with a gradient concentration in the upstream network of the device to induce the expression of GRP78 in the cells cultured in the downstream before the addition of VP-16. The expression of GRP78 was detected by immunofluorescence, the apoptosis for the cells treated by VP-16 was assessed morphologically by 4′,6-diamidino-2-phenylindole (DAPI) staining. The results indicated that the expressions of GRP78 increased greatly for the cells under the induction of A23187 with a dose-depended manner, while the percentage of apoptotic cells decreased significantly after being treated by VP-16. Our results from this study confirmed the role of GRP78 played in the chemotherapy resistance to VP-16 in SK-MES-1 cell line, suggesting that the integrated microfluidic systems may be an unique approach for characterizing the cellular responses.

1. Introduction

Lung cancer is the leading cause of cancer death in human beings all over the world. The major clinical strategy for administration of lung cancer with the advanced stages is chemotherapy. However, the success of the treatment is limited by the intrinsic or acquired resistance of cancer cells to chemotherapy. The stress conditions that induce glucose regulated proteins (GRPs), a major family of stress proteins, include glucose starvation, hypoxia and low pH, all of which are normally observed in solid tumors [1–3]. These conditions could be an important mechanism for the resistance to anticancer drug in solid tumors.

Glucose Regulated Protein-78 (GRP78), a representative member of GRPs family in the tumor cell niche, plays an important role in the chemotherapy resistance in many tumors including breast, ovarian, colon and bladder cancer. However, little is known about the function of GRP78 in human lung cancer chemotherapy resistance. The conventional methods for the in vitro detection of GRP78 mainly include immunoprecipitation, Western blotting, and gel electrophoretisis on the cells cultured in flasks [4,5]. These processes involve a relatively long time, troublesome liquid-handling procedures and large quantities consumption of reagents; some require labor-intensive purification of the protein and complex analysis steps.

Microchip-based systems widely known as micro-total-analysis-systems (μ-TAS) or lab-on-a-chip have been spreading rapidly [6,7]. Many applications, including flow-injection analyses, solvent extractions, and microreactors have been demonstrated [8,9]. In these systems, full advantage was taken of the scale merits of the microspace, such as a short diffusion distance, a large specific interface area, a rapid and efficient reaction. Microchip techniques also appear to provide some advantages for cellular biological analysis systems [10,11], because the scale of the liquid microspace inside a microchip is fitted to the size of the cells. Besides, rapid and sensitive immunoassay systems for protein analyses with these systems have been demonstrated as well [12,13].

* Corresponding author at: Department of Respiratory Medicine, The Second Hospital Affiliated to Dalian Medical University, 467 Zhongshan Road, Dalian 116027, China. Tel.: +86 411 86110151; fax: +86 411 86110151.
E-mail address: wqdmu@yahoo.com.cn (W. Qi).

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In this work, an integrated microfluidic gradient chip was used as a platform to analyze the correlation between the expression of GRP78 and the resistance to anti-cancer drug VP-16 (etoposide) in human squamous carcinoma cell line SK-MES-1. Herein, A23187, an agent of calcium ionophore, a highly potent endoplasmic reticulum stress inducer, was used to induce the expression of GRP78. Also, the potential applications of the microchip system on the research of cell culture and cell function have been investigated.

2. Materials and methods

2.1. Microfluidic gradient chip fabrication

The microfluidic gradient chip was composed of an upstream concentration gradient generator (CGG) and a downstream cell culture module (Fig. 1). The design of the CGG was based on the work previously presented by Jeon et al. [14]. The formula of the concentration interval from channel 1 to channel 8 which generated by CGG in theory is (drug concentration$_{max}$ − drug concentration$_{mix}$)/7. The cell culture module, composed of an array of cell culture chambers, was integrated with the CGG unit, which was similar to the work described by Thompson et al. [15]. Three chambers in row were connected by channels for the performance of three independent cell groups. The microfluidic gradient chip was fabricated in polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI, USA) by using standard soft lithography methods and rapid prototyping techniques [16,17]. The PDMS microfluidic network was irreversibly bonded to a glass slide assisted by oxygen plasma surface treatment (150 mTorr, 50 W, 20 s), creating an optically transparent device for cell culture and protein expression profiling. Dimensions and layout of the chip was shown in Fig. 1.

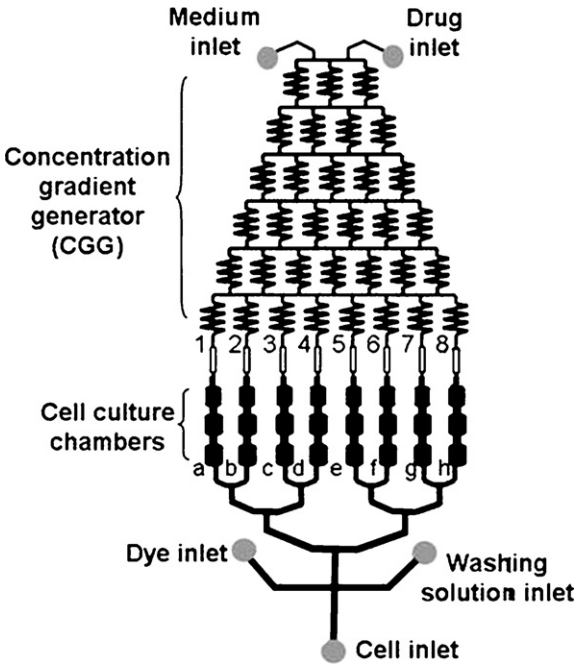

Fig. 1. Layout of the integrated microfluidic device mainly composed of an upstream gradient network (CGG) and the downstream cell culture chambers. The microfluidic channels in the upstream dilution module are 400 μm in length, 50 μm in width and 50 μm in height and generate several concentrations of the stimulus by continuous-flow diffusive mixing of adjacent laminar flow streams. The various concentrations are delivered to the downstream array of culture chambers, each 800 μm long, 500 μm wide and 100 μm in height.

2.2. Cell culture

Prior to cell culture, the PDMS microchip was treated as follows: First, the chamber was washed with dehydrated alcohol, and water, successively. Next, the chip was autoclaved at 120 °C for 15 min. The culture chamber was filled with poly-L-lysine solution (0.01%, m/v) (Sigma–Aldrich Co., St. Louis, MO, USA) for 1 h in order to coat its inner surface. The human lung squamous carcinoma cell line SK-MES-1 was obtained from ATCC (Manassas, VA, USA). The cells were maintained in minimum essential medium (MEM) with sodium pyruvate (1 mM) supplemented with fetal bovine serum (FBS) (10%, v/v)–penicillin (0.2 mM)–streptomycin (0.2 mM) (200:1:1, v/v/v) (pH* 7.4) in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. After being cultured in flasks at 60–70% confluence, the cells were digested with trypsin–PBS and resuspended, then they were injected into the microfluidic device at 5×10^6 cells/mL via the downstream cell inlet by syringe pump. The total injected volume was 10 μL. After incubated for 24 h, the viabilities of the cells were detected by a trypan blue exclusive assay [18]. All the experiments were repeated at least three times.

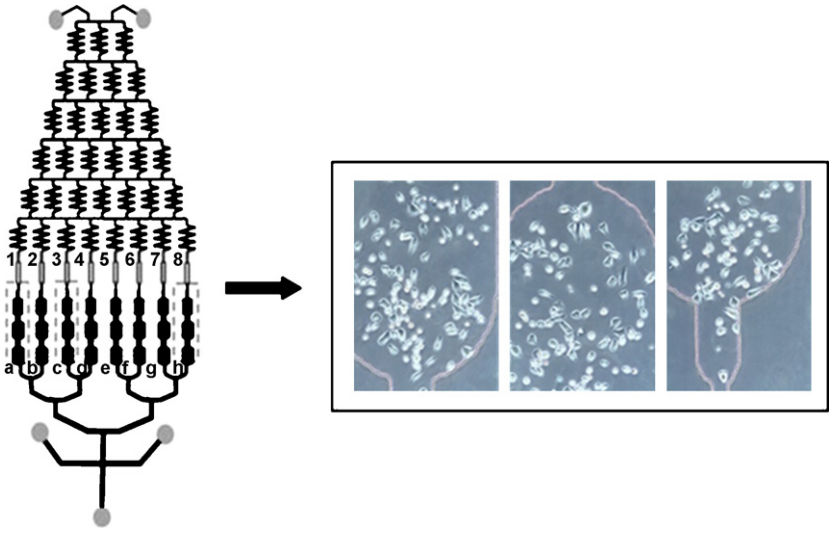

Fig. 2. The SK-MES-1 cells in cell culture chambers were incubated under stable conditions for 24 h. The SK-MES-1 cells could be successfully maintained in the microfluidic device. The viability of cells maintained in the culture chambers was almost unaffected prior to the treatment.

2.3. Immunofluorescence assay of GRP78 protein on PDMS chip

The culture media without and with A23187 at 6 μM were simultaneously infused into the microfluidic device by the syringe pump through the two upstream inlets of the CGG after the cells grown to 50% confluence. Within 30 s, solutions of A23187 with gradient concentrations were established. The device was then kept in an incubator at 37 °C, 5% CO_2 and 100% humidity for 24 h. Then the cells were washed with PBS (pH 7.4, 0.1 M), fixed with paraformaldehyde–PBS (4%, m/v), permeablized in Triton X-100–PBS (0.1%, v/v) and bovine serum albumin–PBS (5%, m/v) before the detection of GRP78 with immunofluorescence. The media with anti-GRP78 (C-20) goat polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a dilution of 1:500 (v/v) were introduced into the culture chamber at 4 °C over night, then, the cells were stained with anti-goat FITC green-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) at a dilution of 1:200 (v/v) at 37 °C for 1 h. Cell images were subsequently captured with fluorescence microscope (IX-71; Olympus Optical Co., Tokyo, Japan) and analyzed using Northern exposure image analysis/archival software (Mississauga, Ontario, Canada).

2.4. Analysis of apoptosis

After induction by A23187 at different concentrations for 24 h, the cells in microfluidic chambers were treated by VP-16 at 30 μM for 6 h. Then, the cells were fixed with 4% paraformaldehyde at room temperature after being cultured in fresh medium for another 48 h. After these, 4′,6-diamidino-2-phenylindole (DAPI, Sigma Chemical Co.), a DNA-binding blue fluorescent dye at a concentration of 1 μg/mL were perfused into the culture chambers to incubate the cells for 30 min. The cells with stained nuclei were visualized and photographed using a Zeiss fluorescence microscope (IX-71; Olympus Optical Co., Tokyo, Japan). Apoptotic cells were morphologically

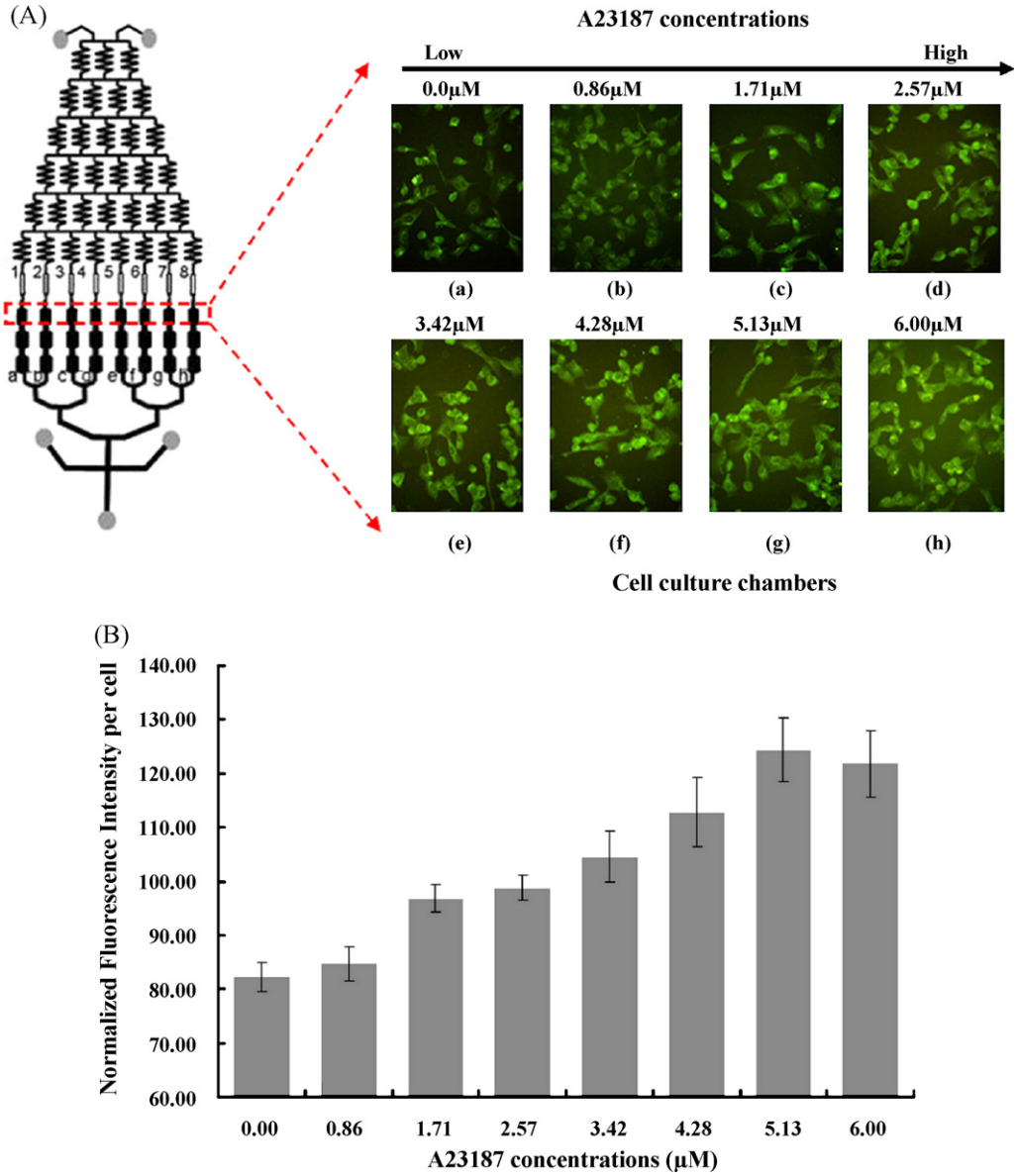

Fig. 3. The expression of GRP78 at protein level by immunofluorescence in the SK-MES-1 cells. (A) Cells were treated with A23187 for 24 h and observed by the fluorescence microscope (IX-71; Olympus Optical Co., Tokyo, Japan) (×400). The fluorescent indicated the GRP78 in the cytoplasm. (B) The average expression of GRP78 in per cell reflected by normalized fluorescent intensities increased with the concentrations of A23187. The normalized fluorescent intensities per cell were determined by the number of cells in a region divided by the fluorescent intensity in that region.

defined by cytoplasmic and nuclear shrinkage and by chromatin condensation or fragmentation [19].

2.5. Image analysis

Fluorescent images were captured and analyzed using fluorescence microscope. The excitation wavelengths were 488 nm for FITC, but 358 nm for DAPI. The detection wavelengths were set at 500–535 nm for FITC, but 460 nm by the grating system. The average fluorescence of each cell region was corrected for medium and device fluorescence by subtracting the average local background fluorescence [20].

3. Results and discussion

3.1. Validation of the integrated microfluidic gradient chip device performance

The performance of the integrated microfluidic gradient chip device was validated prior to the induction of A23187. It has been proved that there was good coherence between the experimental and theoretical data in the CGG [20]. Therefore, the concentrations of A23187 formed in channels 1–8 could be obtained by theoretical calculations. In the experiments, the two inlets were supplied with culture media containing 0.00 and 6.00 μM of A23187, thus, the

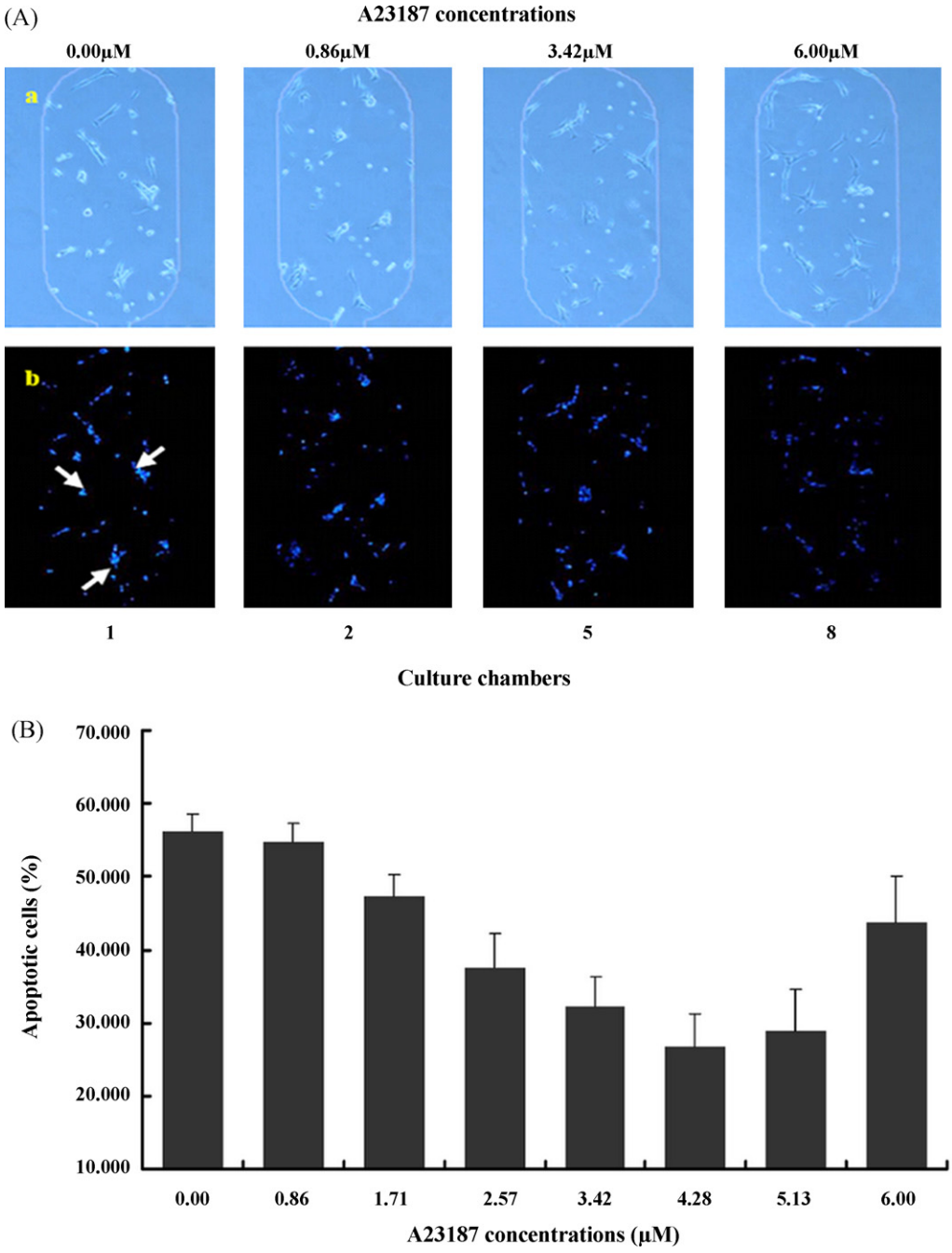

Fig. 4. (A) Fluorescent microscopic analysis of apoptotic cells stained with DAPI after treatment with VP-16 for SK-MES-1 cells with induction of different concentrations of A23187. (a) SK-MES-1 cells in the culture chambers after staining. (b) Imaging of nuclear morphology within SK-MES-1 cells using fluorescent microscopy. Shrunken nuclei of DAPI-stained cells (white arrows) are hallmarks of apoptosis (×100). (B) The percentage of apoptotic cells with DAPI staining was dose-dependent with the concentrations of A23187. The cells were treated with DAPI for 30 min and observed by the fluorescence microscope (×100). All the experiments were repeated at least three times.

concentrations in the eight channels were 0.00, 0.86, 1.71, 2.57, 3.42, 4.28, 5.13, 6.00 μM, respectively. Since the concentration of A23187 in channel 1 is 0 μM, the cells in the corresponding downstream culture chambers would be served as a control group. Therefore, a wide range of A23187 solutions with well-defined and different concentrations from 0.00 to 6.00 μM can be automatically generated by only a binary input of a blank solution and an A23187 solution at concentration of 6.00 μM. Our finding from this study by using the integrated microfluidic gradient chip device was also supported by recent studies. Lately, the reports from other groups also suggested that this kind of integrated microfluidic gradient device can be used in many researches including quantitative analysis of cancer cell chenmotaxis [21] and high content screening by the multiparametric measurement of cellular in response to several drugs [22]. However, most of the reports focused on the cell line, few were involved in the tissue, it may be caused by the complicated conditions for the tissue culture.

3.2. Microfluidic cell culture

Fig. 2 showed SK-MES-1 cells cultured in the microchannels of PDMS chip for 24 h with good spreading and attachment to chip. The results with less than 1% of dead cells (data not shown) measured by trypan blue exclusion assay suggested that the cell culture chambers of the microdevice provide a suitable environment for cell maintenance.

3.3. Expression of GRP78 protein

Fig. 3A shown the expression of GRP78 for the cells growing in culture chambers treated with A23187 at eight concentrations for 24 h. GRP78 displayed a perinuclear, reticular pattern of distribution in the cells. The normalized fluorescent intensities for per cell were shown in Fig. 3B. As it shown, when the concentration of A23187 increased from 0.86 to 6.00 μM, the average fluorescent intensity for per cell increased obviously with a dose-depended manner. All these meant A23187 could induce the expression of GRP78 effectively in SK-MES-1 cell line.

3.4. Correlation between overexpression of GRP78 and drug-resistance to VP-16

In order to know whether the elevation of GRP78 induced by A23187 is associated with the resistance to VP-16 in SK-MES-1 cells, the specific features of apoptosis, such as chromatin condensation and DNA fragmentation were detected with DAPI staining for the cells being induced by VP-16 (Fig. 4A). As shown in Fig. 4B, the percentage of apoptosis for the A23187-pretreated cells decrease greatly with a concentration-dependent manner, especially when the concentrations of A23187 ranging from 0.00 to 4.28 μM; whereas when the concentration was higher than 4.28 μM, the decrease was not as significantly as the former. These results suggested that the overexpression of GRP78 could suppress VP-16 induced apoptosis in SK-MES-1 cell line, which meant GRP78 had the correlation to the resistance to VP-16 in human lung squamous carcinoma.

Consistent with this study using the integrated microfluidic device, our previous work with conventional methods also showed that GRP78 was significant associated with VP-16 sensitivity in lung cancer cell line [23], which means the microfluidic device can be used in the analysis of cell contents and drug resistance with a good correlation with those by the conventional methods with cells cultured in flasks. Compared to the conventional methods, the microfluidics-based platform shown many advantages due to its capabilities of miniaturizing and integrating various operations by flexible and scalable approaches. In this study, the immunofluorescence assay of GRP78 and apoptosis detection morphologically for the cells in PDMS chips involved simple liquid-handling procedures and low consumption of reagents. The whole processes from cell culture to the analysis of protein expression and apoptosis were done continuously and conveniently without time wasting. In addition, the flexibility of a rapid prototyping technique for the PDMS chip fabrication allows the possibility of further increasing and modifying the functional units for simultaneously testing hundreds samples of different stimulators and cells, which makes it an attractive platform for medium-throughput and medium-content drug screening at cellular level. All these suggested that the microfluidic chip system maybe an ideal approach for cell-based biological and medical studies. Its benefit to extensive biomedical research is required to be further estimated.

4. Conclusion

This study demonstrated that overexpression of GRP78 induced by calcium ionophore A23187 directly conferred resistance to VP-16 induced apoptosis in human lung squamous carcinoma SK-MES-1. Quickly concentration gradient generation, simple liquid-handling procedures, low reagents consumption and medium-throughput analysis of the GRP78 and apoptosis detection morphologically were attained by the microchip-based system. This system was valuable in the cell culture and the detection of cell components and deserved to be evaluated further.

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