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Characterizing Cell Death Events Using a Microfluidics-based Method

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Abstract

Cell death is the ultimate endpoint of the cellular life cycle, and the ability to distinguish different types of cell death (e.g., apoptosis and necrosis) has been a subject of intensive research. This paper demonstrates the suitability and efficiency of a microfluidics-based method for characterization of cell death events. A multicompartmented microfluidic device was used to examine the types of cell death induced by cadmium ions at various concentrations. Annexin V-FITC in combination with propidium iodide was used to distinguish between viable, apoptotic, and necrotic cells. The microfluidics-based method facilitates the performance of analysis of cell death on the basis of membrane alteration as well as morphological discrimination. In addition, this method shows high sensitivity and specificity, as evidenced by the quantification of the ratio of apoptotic and necrotic cells in the total population of cells.

Keywords: Cell death, Apoptosis, Necrosis, Microfluidic multicompartments, Cadmium ion

Introduction

Cell death is the ultimate endpoint of the cellular life cycle and is generally categorized into three distinct types: apoptosis (type I), autophagic cell death (type II), and necrosis (type III)[1,2]. Apoptosis is often used to describe the physiological death of mammalian cells. Cells undergoing apoptosis generally show the characteristic features: round or oval-shaped appearance, chromatin condensation (pyknosis), nuclear fragmentation (karyorhexis), overall shrinkage of cells, plasma membrane blebbing, sustained presence of phosphatidylserine in the outer leaflet of the plasma membrane, and the formation of apoptotic bodies[1-3]. Autophagic cell death is manifested by a massive accumulation of two-membrane autophagic vacuoles in the cytoplasm[1,2]. In contrast, necrosis has been described as accidental and uncontrolled cell death due to extreme physicochemical stress on the cell, such as heat, osmotic shock, mechanical stress, freeze/thawing and high concentration of hydrogen peroxide[3-5]. It is generally characterized by cytoplasmic swelling, mechanical rupturing of the plasma membrane, and dilatation of cytoplasmic organelles[1-5].

It is very important to distinguish necrosis from other types of cell death, as it is often associated with unwarranted cell loss in human pathologies and can lead to local inflammation due to liberation of intracellular factors from dead cells that alert the innate immune systems[4,5]. However, there are only a few known biochemical markers that unambiguously discriminate necrosis from apoptosis. Recently, Annexin V has been widely used as a potent discriminator between viable and apoptotic cells[6,7]. Due to its high affinity for phosphatidylserine residues, it specifically binds to cells that have compromised plasma membrane integrity[6,7]. As a consequence, Annexin V, as a FITC conjugate (Annexin V-FITC) in combination with propidium iodide (PI), is effectively used to distinguish between viable, apoptotic, and necrotic cells[8]. Notably, this assay is quite easy to perform, as it does not require prolonged incubation periods. Analysis can be carried out using a fluorescence microscope for adherent cells and a flow cytometer for cells in suspension[6-8].

Flow cytometric analysis is a popular method as it provides quantitative results related to cell death events with high sensitivity and specificity[6,7]. However, there are some problems associated with this method, including high cost, infrequent accessibility, and the requirement of suspension cultures[7]. Simple but efficient approaches using laboratory light and fluorescent microscopes are required in the analysis of types of cell death. The development of miniaturized high-throughput analytical devices and their integration to create new analytical systems is one of the directions being taken to address the shortcomings associated with conventional technology[9].

Recently, microfabricated microfluidics-based technology has been extensively used in numerous biolo-

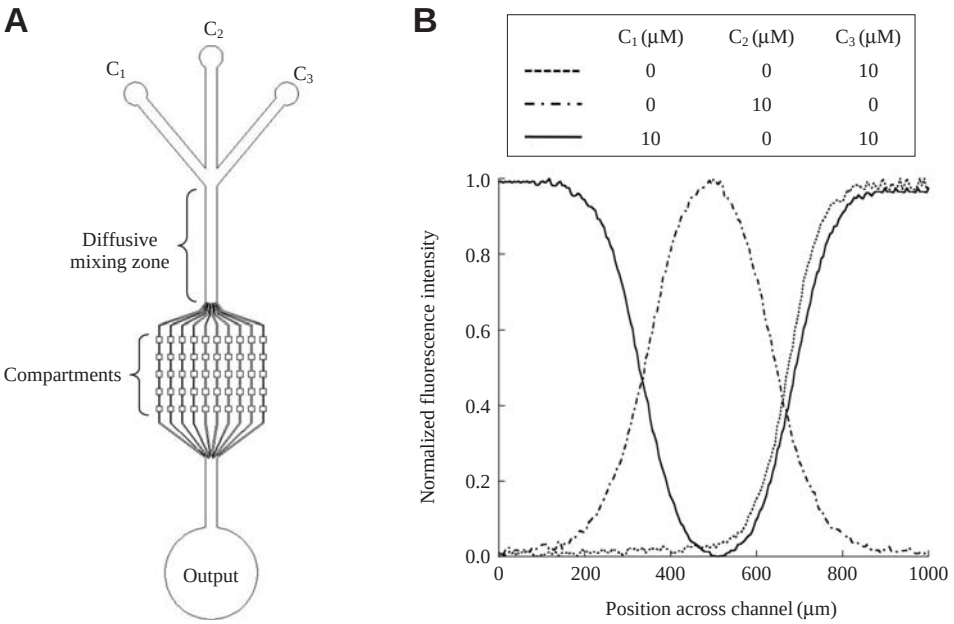

Figure 1. Schematic representation and characterization of the multicompartmented microfluidic device. (A) The microfluidic network design consists of an upstream mixing zone and downstream cell culture compartments. (B) Fluorescence intensity profile taken across the main wide channel (5 mm long, 1 mm wide, and 0.1 mm high) of the mixing zone with various combinations of input solutions using 0.0 and 10.0 μM of fluorescein ions.

gical and toxicological applications, such as cell migration[10], gene expression[11,12], differentiation[13], and cytotoxicity assay[14]. For instance, Ye *et al*.[15] efficiently used a microfluidic platform for characterization of doxorubicin-induced apoptosis in HepG2 cells. Qin *et al*.[9] described the capabilities of microfuidic devices for the comprehensive investigation of apoptosis of cells, demonstrating the applicability of devices for the analysis of the types of cell death based on morphological discrimination.

This work describes the development of a microfluidics-based method for the analysis of cell death events, particularly apoptosis and necrosis, using an Annexin V-FITC/PI assay. A multicompartmented microfluidic device was fabricated for the generation of a well-defined concentration gradient of a toxicant-cadmium ion in the present study. The cell death events of BALB/3T3 fibroblast cells were monitored based on morphological discrimination by DIC microscopic images as well as high-resolution fluorescence images induced by Annexin V-FITC combined with PI.

Results and Discussion

Observation of Morphological Changes

A dose-dependent morphological change was observed following treatment of cadmium ions for 12 h, as examined and photographed by a DIC microscope from 10 compartments (Figure 2A). Morphological alterations include typical features of apoptosis, such as rapid shrinkage of cells, membrane blebbing, rounding of the cells, and loss of cell adhesion. These types of morphological changes were not observed in the control cells. In the time course study, cells treated with 50.0 μM cadmium ions were observed through live cell imaging for 6 h and displayed characteristics of the early stages of apoptotic response in the initial hours (0-2 h) of treatment, as manifested by profound shrinkage of cells, increased cell granularity, irregularity of cell outline, and fragmentation[16]. In the late hours (2-6 h) of treatment, cells elicited the characteristics of the late stages of apoptotic response, as evidenced by the formation of apoptotic bodies[16].

Analysis of Cell Death Events

Cell death induced by cadmium ions was characterized by staining cells with Annexin V-FITC and PI. It is evident that changes occur on the cell surface in the early stages of apoptosis[17]. The externalization of phosphatidylserine from the inner face of the plasma membrane to the outer leaflet is considered to be a primary event of apoptotic cell death[7]. Annexin V, a Ca^{2+}-dependent phosphatidyl-binding protein, preferentially binds to phosphatidylserine. Thus, this protein can be used to identify the early stages of apoptosis. Fluorescent probes combining Annexin V-FITC and PI can effectively identify the late stages of apoptosis, as loss of membrane integrity occurs at these stages. Necrotic cells can be detected by staining with PI alone.

As shown in Figure 2B, cells in the early stages of apoptosis exposed phosphatidylserine to the outer leaflet, while maintaining membrane integrity [Annex-

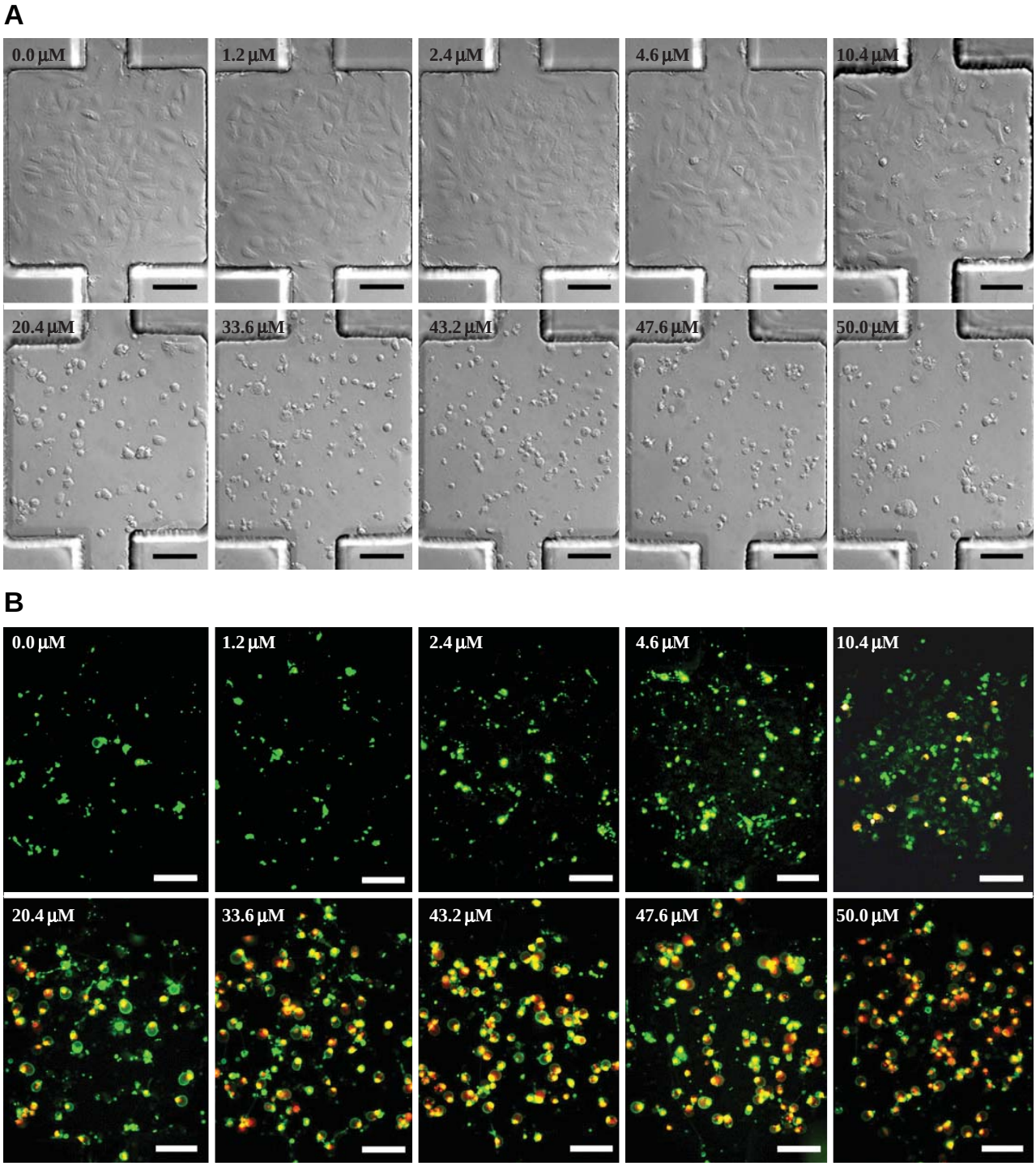

Figure 2. (A) Morphological changes in BALB/3T3 cells. DIC images of cultured fibroblast BALB/3T3 cells treated with a gradient flow of growth media containing 0.0 and 50.0 μM cadmium chloride for 12 h. (B) Assessment of apoptotic and necrotic cells. Cells, pretreated with a gradient flow of growth media containing 0.0 and 50.0 μM cadmium chloride for 12 h, were stained with Annexin V-FITC/PI to detect distinct phases of apoptotic cells and necrotic cells. Scale bars: 100 μm.

in V-FITC(+)/PI(−)]. In the late stages of apoptosis, the cellular membrane integrity was lost [Annexin V-FITC(+)/PI(+)]. Ultimate breakdown of the membrane in necrotic cells was observed [Annexin V-FITC

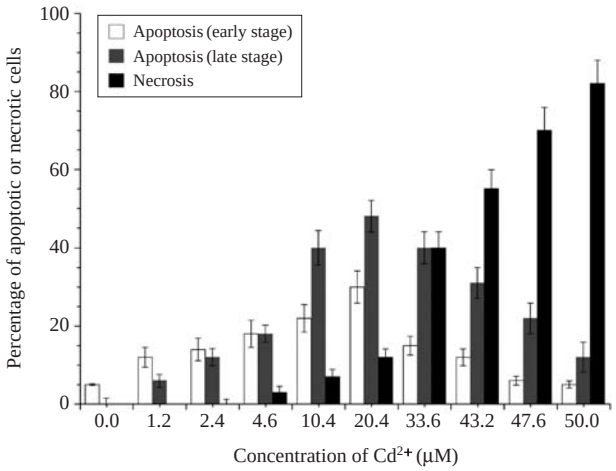

Figure 3. Quantification of cadmium ion-induced apoptotic and necrotic cells. Results are expressed as percentages of apoptosis/necrosis with regard to the total population of cells. Data are represented as mean ± SD of three independent experiments.

(−)/PI(+)]. Fluorescent images were used to quantify the ratio of apoptotic and necrotic cells in the total population of cells. Furthermore, the percentages of different stages of apoptotic and necrotic cells were evaluated in a dose-dependent manner (Figure 3). At a lower range of cadmium ion concentrations (0-20.4 μM), the early and late apoptosis of cells were the main events of cell death, and the maximum values of early and late apoptotic cells were observed at 20.4 μM of cadmium ions. The percentages of early and late apoptosis of cells were estimated to be around 30% and 48%, respectively, as compared to 5% for the control. Above 20.4 μM, apoptotic cell death events diminished very significantly and necrosis became a more prominent event. The estimated value of necrotic cells increased by 82% at 50.0 μM of cadmium ions as compared to the control.

The microfluidics-based method developed in this work offers distinct advantages over the conventional flow cytometric methods. First, it facilitates the examination of death events of adherent cells. Second, it allows the detection of the death event of a single cell or a small population of cells based on location and morphology. Third, it utilizes the advantages of a live cell imaging system very efficiently, and therefore allows the real-time monitoring of dynamic cell death events. In comparison to other microfluidic approaches for the analyses of cell death[9,15], the method described here also has similar features such as simplicity of design, analysis of death events based on morphological and membrane alterations, single-cell analysis, and dosage-dependent cellular analysis. In addition, it is anticipated that the proposed microfluidics-based method could be useful for high-throughput apoptosis assays for drug discovery, cytotoxicity screening, and precise understanding of biological pathways.

Conclusions

The suitability and efficiency of a microfluidics-based method for the analysis of cell death events were demonstrated using a multicompartmented microfluidic device. This integrated microfluidic platform facilitates the performance of analyses of cell death events on the basis of observation of membrane alteration as well as morphological changes. Cell death events induced by cadmium ions were examined using a high-throughput multicompartmented microfluidic device at various concentrations. This method shows high sensitivity and specificity as evidenced by the quantification of the ratio of apoptotic and necrotic cells in the total population of cells. In addition, the microfluidics-based method allows the examination of death events of adherent cells, and the device has tremendous capabilities for application in the design of cytotoxicity experiments.

Materials and Methods

Fabrication of Device and Functional Validation

The multicompartmented microfluidic cell culture device was fabricated in poly(dimethylsiloxane), PDMS (Dow Corning, Sylgard 184), using rapid prototyping and soft lithography according to previously reported procedures[18,19]. The device is composed of an upstream microfluidic diffusion diluter (μDD) and a downstream cell culture module, as shown in Figure 1. The μDD, based on the fluidics of diffusional mixing previously described by Holden *et al.*[20], consists of a mixing zone associated with three distinct inputs, generating ten outlet concentrations into a downstream array of cell culture compartments. The cell culture module, based on the work described by Thompson *et al.*[12], consists of an array of 500 μm × 500 μm cell culture compartments where BALB/3T3 fibroblast cell lines were grown in individual compartments of a single microfluidic network. The concentration profiles of toxicants in the microfluidic channels were characterized by injecting fluorescein ions with comparable molecular weight to that of hydrated cadmium ions. The concentrations entering the downstream cell culture compartments were quantified using the steady and calibrated gradient profile of the fluorescein ions[15].

Cell Culture, Live Cell Imaging, and Apoptosis Assay

To avoid the attachment and growth of cells on undesired regions, octadecyltrichlorosilane (OTS, Aldrich) was selectively coated over the μDD network. The mouse embryonic fibroblast cell line BALB/3T3 (Korean Collection for Type Cultures, KCTC) was cultured in low glucose Dulbecco's modified Eagles medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin-streptomycin (Sigma) in a humidified incubator (Sanyo Electric) at 37˚C with 5% CO_2. In the case of perfusion-controlled microfluidic experiments, the device was first filled with growth medium overnight to equilibrate PDMS channels with the medium. A suspension of BALB/3T3 cells (4×10^6 cells/mL; 200 μL) was introduced into the device outlet and then placed in a humidified incubator for 24 h to allow for proper cell attachment and spreading. The microfluidic device containing confluent fibroblast cells was placed inside an environmental chamber (37˚C and 5% CO_2) on an inverted microscope, an Olympus IX71 equipped with a DP71 CCD camera. For a long-term cell culture, time-lapse images were acquired on a motorized microscope stage (Prior Scientific Inc.) every 5 min for 12 h using a 20× differential interference contrast (DIC) objective lens. Treatment was delivered to cultured cells by performing simultaneous infusion of culture media with and without 50.0 μM cadmium chloride (Aldrich) using a syringe pump (Harvard Apparatus) through the inlets of μDD. Stimulations with a constant flow rate of cadmium ions were performed at 0.5 μL/min. After completion of the treatment, PBS was introduced into the channel to gently rinse the cells, and an apoptosis assay was subsequently performed. The apoptotic and necrotic cells were determined by staining with an Annexin V-FITC/PI apoptosis detection kit (Sigma). After gentle washing with PBS, cells were incubated with Annexin V-FITC and PI labeling solution for 10 min. Cells were washed with a binding buffer, and analyzed using a fluorescence microscope with appropriate filter settings. Three independent experiments were performed in each case for a quantitative analysis of the cells.

Acknowledgements

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