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A high-throughput fluorescence resonance energy transfer (FRET)-based endothelial cell apoptosis assay and its application for screening vascular disrupting agents

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ABSTRACT

In this study, we developed a high-throughput endothelial cell apoptosis assay using a fluorescence resonance energy transfer (FRET)-based biosensor. After exposure to apoptotic inducer UV-irradiation or anticancer drugs such as paclitaxel, the fluorescence of the cells changed from green to blue. We developed this method into a high-throughput assay in 96-well plates by measuring the emission ratio of yellow fluorescent protein (YFP) to cyan fluorescent protein (CFP) to monitor the activation of a key protease, caspase-3, during apoptosis. The Z' factor for this assay was above 0.5 which indicates that this assay is suitable for a high-throughput analysis. Finally, we applied this functional high-throughput assay for screening vascular disrupting agents (VDA) which could induce endothelial cell apoptosis from our in-house compounds library and dioscin was identified as a hit. As this assay allows real time and sensitive detection of cell apoptosis, it will be a useful tool for monitoring endothelial cell apoptosis in living cell situation and for identifying new VDA candidates via a high-throughput screening.

1. Introduction

Endothelial cells line the interior surface of the blood vessels. As located at the interface between the blood and tissue, endothelial cells are exposed to various stimuli with the potential to promote or prevent apoptosis. Physiological endothelial function involves a homeostasis between pro- and anti-apoptotic signals, and perturbation of this balance may contribute to the occurence of diverse vascular diseases [1]. Endothelial cell apoptosis has been associated with cardiovascular pathologies, in particular with atherosclerosis and thrombosis [2,3]. On the other side, the angiogenic factors such as vascular endothelial growth factor (VEGF) can not only inhibit endothelial cell apoptosis but also stimulate them to form capillary tubes which are essential for the growth and metastasis of solid tumors [4]. Endothelial cells have been considered as the targets for tumor therapy. Therefore, a sensitive endothelial cell apoptosis assay is quite useful for understanding the role of endothelial apoptosis in the pathogenesis of different diseases as well as developing the therapeutic agents intervening in this process.

During cell apoptosis, the activated caspase-3 is the most important execution protease, whose enzymatic activity is suitable for the evaluation of apoptosis. Caspase-3 activity can be detected in live cells using fluorescence resonance energy transfer (FRET)-based biosensors [5–7]. In addition, our group developed a FRET biosensor C3-based high-throughput screening method for anticancer drugs discovery [8–10]. C3 is a recombinant substrate protein for caspase-3 and consisted of three parts: a donor cyan fluorescent protein (CFP), a 16 amino acid peptide linker containing the caspase-3 cleavage sequence Asp-Glu-Val-Asp (DEVD), and an acceptor yellow fluorescent protein (YFP) [7]. Under the normal growth condition, the fluorescent emission energy of excited CFP can be transferred to YFP, resulting in a green fluorescent emission light. However, when caspase-3 is activated by the apoptotic inducers, the linker peptide between CFP and YFP is cleaved, abolishing the FRET effect and resulting in a change of emission light from green color to blue. Thus, by measuring the fluorescence emission ratio of YFP over CFP, we can monitor the kinetics of caspase-3 activation in living cells during apoptosis [7,8]. In the present study, we established a stable endothelial cell line that can constitutively express this FRET-based caspase sensor, and furthermore, we developed a high-throughput assay based on this cell line.

Subsequently, we applied this high-throughput endothelial cell apoptosis assay for screening vascular disrupting agent (VDA), since the mechanisms of VDA involve directly or indirectly inducing endothelial cell apoptosis. After screening an in-house compounds library, we discovered the herbal compound dioscin with the apoptotic effect on endothelial cells.

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2. Materials and methods

2.1. Generation of HUVEC-C3 stable cell line

The plasmid DNA of biosensor C3 which contains CFP–DEVD–YFP fusion gene was constructed and characterized by our group previously [7]. Human umbilical vein endothelial cell (HUVEC) line was obtained from American Type Culture Collection. HUVEC was maintained in DMEM/F12 (Invitrogen) which was supplemented with 10% fetal bovine serum (FBS). C3 plasmid was introduced into HUVEC by using transfection reagent HilyMax (Dojindo Laboratories), and the stable cell line designated as HUVEC-C3 was established and maintained in DMEM/F12 with 10% FBS and 500 μg/ml geneticin.

2.2. Detection of FRET changes using a fluorescence microscope

HUVEC-C3 cells were grown overnight on a 25 mm round coverslip mounted onto a 35 mm Petri dish with an observation window. Cells were irradiated with UV light in a biosafety cabinet for 3 min to induce apoptosis. Then HUVEC-C3 cells were incubated for different periods to observe the time-dependent FRET changes. We also studied the effects of 24 h incubation of an anticancer drug paclitaxel (30 nM, Sigma–Aldrich) on FRET changes. After treatments with apoptotic inducers, the fluorescent images of living cells were obtained using an inverted fluorescence microscope (Zeiss Axiovert 100). The cells were excited using an excitation filter (436 ± 10 nm) plus a diachronic mirror of 455 nm. The emission images of YFP (535 ± 15 nm) and CFP (480 ± 20 nm) were recorded using SPOT CCD camera (Diagnostic Instruments).

2.3. Determination of FRET changes using a fluorescent plate reader

10,000 HUVEC-C3 cells were seeded into each well of a 96-well plate. After an overnight incubation, cells were irradiated with UV light for 3 min. Then, after different periods of incubation, the plates were read by a Perkin-Elmer Victor^3 plate reader under the excitation wavelength of 440 ± 4 nm. The emission intensity was measured at 486 ± 5 nm for CFP and 535 ± 12.5 nm for YFP. We also studied whether the necrotic inducers including H_2O_2 (5 mM) and Triton X-100 (0.1%) had the effects on FRET. To avoid the interference of auto fluorescence from culture medium or compounds, we used net YFP and CFP readings by subtracting the background fluorescence for data analysis. In this paper, Y/C emission ratio was used to represent the effect of FRET, which equaled to the net YFP reading divided by the net CFP reading from the same well at each time point.

2.4. Cell viability assay

The MTT assay was used to measure the cell viability after different treatments. At each designated time point, 10 μl of MTT (5 mg/ml, Sigma–Aldrich) was added into each well, and incubated for 4 h. Then, 100 μl of 10% SDS containing 10 mM HCl was added to dissolve the purple crystals which are the products of MTT substrates. After 12 h incubation, the absorbance was measured by a plate reader at 595 nm. The relative cell viability was calculated as $B/A \times 100\%$, where A and B are the absorbance of control and that of the treated cells. CC_{50} value (50% cytotoxicity over the control) is the concentration that can reduce the viability of treated cells by 50% over the control cells and it is used to indicate a compound's cytotoxic effect.

2.5. Caspase-3 activity assay and Western blot analysis

The caspase-3 activity assay was performed by using a caspase-3 fluorogenic substrate Ac-DEVD-AMC (BD Pharmingen) as described before [8]. For Western blot, cell lysate was separated by 12% SDS–PAGE and transferred onto a Hybond ECL nitrocellulose membrane (GE Healthcare). After blocking with 5% non-fat milk for 1 h, the membranes were incubated overnight at 4 °C with different antibodies including mouse-anti-PARP (sc-8007, Santa Cruz), rabbit-anti-β-tubulin (2146, Cell Signaling), and rabbit-anti-GFP (2956S, Cell Signaling). The membranes were then incubated with a horseradish peroxidase-conjugated mouse (170–6516, Bio-Rad) or rabbit (170–6515, Bio-Rad) secondary antibody at room temperature for 2 h, and developed using the ECL SuperSignal West Pico Chemiluminescent Substrate (Pierce) and the signals were recorded by an imaging analyzer LAS-4000 (Fujifilm).

2.6. Data analysis

Data were presented as means ± SD. Statistical difference between different groups were evaluated by one-way ANOVA, and the p value < 0.05 was considered statistically significant. The Z' factor was calculated using the formula $Z' = 1 - [(3\sigma_s + 3\sigma_c)/|\mu_s - \mu_c|]$, where μ_s and μ_c are the average Y/C values from the stimulated (30 nM paclitaxel treated for 72 h) wells and control wells on the assay plate, and σ_s and σ_c are the standard deviations from the stimulated and control wells, respectively.

3. Results

3.1. Real-time monitoring of FRET during apoptosis in living HUVEC-C3 cells

Typical time-course of caspase-3 activation induced by UV-irradiation was shown in Fig. 1. The normal HUVEC-C3 cells appeared in green color. At 6 h after UV-irradiation, some cells began to emit blue fluorescence. Then more blue colored cells were observed after 9–18 h of incubation. At 24 h after UV-irradiation, nearly all the cells emitted blue fluorescence indicating that the FRET effect in those cells was completely abolished due to the cleavage of C3 sensor by caspase-3 (Fig. 1). At the same time, all the blue cells appeared in round-up and shrinkage morphology indicating those cells already died through apoptosis.

3.2. Characterization of FRET in 96-well plates

In order to develop a high-throughput screening assay for monitoring caspase-3 activation in endothelial cells, we studied the FRET effect in 96-well plates. After 3 min UV-irradiation, we observed that Y/C emission ratio began to decrease after 3 h and reduced significantly after 6 h incubation since UV-irradiation. Within 12 h after UV-irradiation, the Y/C ratio reduced from its maximum value of 5.75 ± 0.63 to its minimum level of 2.09 ± 0.11. At this time, the cell viability was measured to be 15.0 ± 0.7% by MTT assay (Table 1).

Since sensor C3 was designed to detect caspase-3 activation during cell apoptosis, it should specifically detect apoptosis but not necrosis. We confirmed this property by using two necrotic inducers, H_2O_2 (5 mM) and Triton X-100 (0.1%). We observed that cell viabilities reduced almost to zero within 6 h treatment of these two necrotic inducers (Table 1). However, no clear Y/C emission ratio change was detected from cells treated with either 5 mM H_2O_2 or 0.1% Triton X-100 at any examined times (Table 1). Though HUVEC-C3 cells were lysed by Triton X-100, the YFP and CFP emission

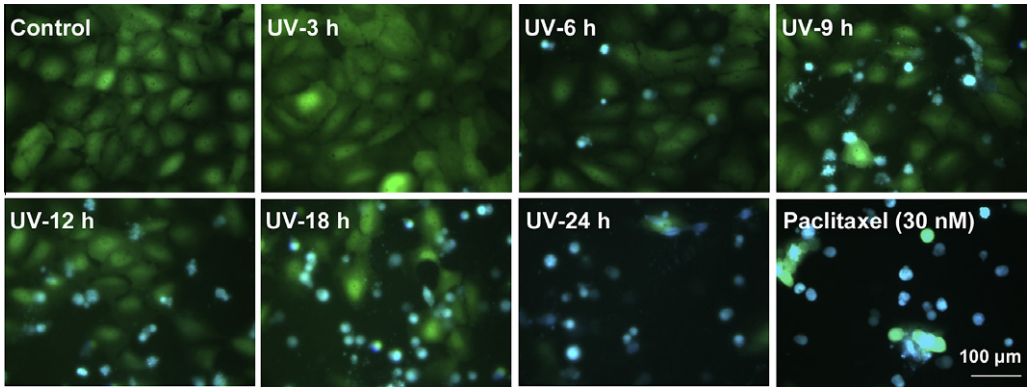

Fig. 1. The fluorescence of HUVEC-C3 cells changes from green to blue in response to cell apoptosis induced by UV-irradiation and paclitaxel. HUVEC-C3 cells seeded on a cover-slip-based observation chamber were treated with 3 min of UV-irradiation followed by different periods of incubation. In addition, HUVEC-C3 cells were treated with paclitaxel (30 nM) for 24 h. The fluorescent images of YFP and that of CFP were separately recorded from the same observation field in the green and blue channels. Then, the YFP and CFP images were merged. The merged color showed a significant blue shift from the cells with shrinkage morphologies and apoptotic bodies, which are the typical features of apoptosis. Scale bar = 100 μm.

Table 1
Y/C emission ratio from HUVEC-C3 was reduced by apoptotic but not necrotic inducers.

Group	Y/C emission ratio	Cell viability (%)
Control	5.75 ± 0.63	100.0
UV-1 h	5.76 ± 0.46	90.8 ± 19.2
UV-3 h	4.93 ± 0.33	75.3 ± 12.5
UV-6 h	3.11 ± 0.23	59.0 ± 6.3
UV-9 h	2.23 ± 0.16	45.2 ± 8.6
UV-12 h	2.09 ± 0.11	15.0 ± 0.7
UV-24 h	2.02 ± 0.19	16.1 ± 1.1
H_2O_2-1 h	5.74 ± 0.56	17.4 ± 4.8
H_2O_2-3 h	5.32 ± 0.61	8.1 ± 3.6
H_2O_2-6 h	5.37 ± 0.77	−1.1 ± 0.2
Triton X-100-1 h	6.02 ± 0.46	0.6 ± 0.1
Triton X-100-3 h	6.19 ± 0.39	0.4 ± 0.1
Triton X-100-6 h	6.28 ± 0.41	0.4 ± 0.1

10,000 HUVEC-C3 cells were seeded into each well of the 96-well plates, and after overnight incubation, the cells were treated with the apoptotic inducer 3 min UV-irradiation, or necrotic inducers H_2O_2 (5 mM) and Triton X-100 (0.1%). The YFP and CFP emission intensities were recorded by the plate reader at the designed time points, and YFP over CFP (Y/C) emission ratios were calculated. At each time point for the treatment groups after fluorescence was measured, the cell viability was determined by MTT assay. Data shown are means ± SD.

from the culture medium suspension could still be measured by the plate reader.

3.3. Confirmation of sensor C3 cleavage during apoptosis induced by UV-irradiation

Before testing whether sensor C3 fusion protein was cleaved into two parts during apoptosis, we first checked whether caspase-3 was activated during UV-induced apoptosis. Results from Fig. 2 show that significant increases of caspase-3 activity and the cleavage of PARP which is the endogenous substrate for caspase-3 were detected only in apoptotic inducer UV-irradiation treated cells, but not in necrotic inducer H_2O_2 treated cells.

We then used GFP antibody to reveal the cleavage of sensor C3. According to the design of this sensor, it can be cleaved by caspase-3 into two parts: CFP–DEVD and YFP, both of which shall be recognized by the GFP antibody. As shown in Fig. 2B, sensor C3 appeared as an intact protein in the control cells. Some of the C3 was cleaved at 3 h, and more cleavages were observed between 6 and 12 h after the UV-irradiation. The temporal profile of sensor cleavage is similar to that of PARP, indicating that the cleavage of sensor C3 can represent the activation of caspase-3 inside cells during apoptosis. No cleavage of sensor C3 was observed after necrotic inducer H_2O_2 (5 mM) treatment, thus further confirming the specificity of sensor C3 in detecting caspase-3 activation during apoptosis.

3.4. HUVEC-C3 cells can detect caspase-3 activation during apoptosis induced by paclitaxel

To evaluate whether HUVEC-C3 cells can be used in a high-throughput assay for identifying apoptotic inducers as potential anticancer agents to endothelial cells, we tested this system with a well-known anticancer drug paclitaxel. Paclitaxel can arrest cells in mitosis by preventing microtubule disassembly and subsequently induce these mitotic arrested cells into apoptosis. The MTT assay revealed that paclitaxel inhibited the viability of HUVEC-C3 cells in a dose-dependent manner (Fig. 3A). The CC_{50} value of paclitaxel was calculated to be 1.20 ± 0.04 nM. We also measured the concentration of paclitaxel that can reduce 50% of the FRET effect or cause 50% of Y/C emission ratio reduction using our FRET sensor-based assay and the value was 0.71 ± 0.11 nM (Fig. 3B), indicating our FRET sensor-based method is more sensitive than the MTT assay. In addition, we showed that 30 nM paclitaxel produced a much faster reduction of the Y/C emission ratio than 3 nM paclitaxel (Fig. 3C). This result demonstrates that our FRET sensor-based assay can be used to compare the potency of agents that can induce apoptosis by activating caspase-3. When the cells were pre-treated with a pan-caspase inhibitor Z-VAD-FMK (40 μM, Merck) for 2 h, the Y/C emission ratio reduction induced by 36 h incubation of 30 nM paclitaxel was significantly inhibited (Fig. 3D). This result also demonstrated the selectivity of this FRET sensor-based caspase assay.

Z' factor is a simple statistical parameter to evaluate the quality of a high-throughput screening assay [11]. We defined the 30 nM paclitaxel treatment for 72 h as the stimulated group and calculated the Z' factor value (Fig. 3E). The Z' value for this assay was measured to be 0.66. Since Z' value larger than 0.5 is considered as the standard for a high-throughput screening method, we can conclude that the caspase sensor-based assay developed in this study is a reliable and reproducible high-throughput method.

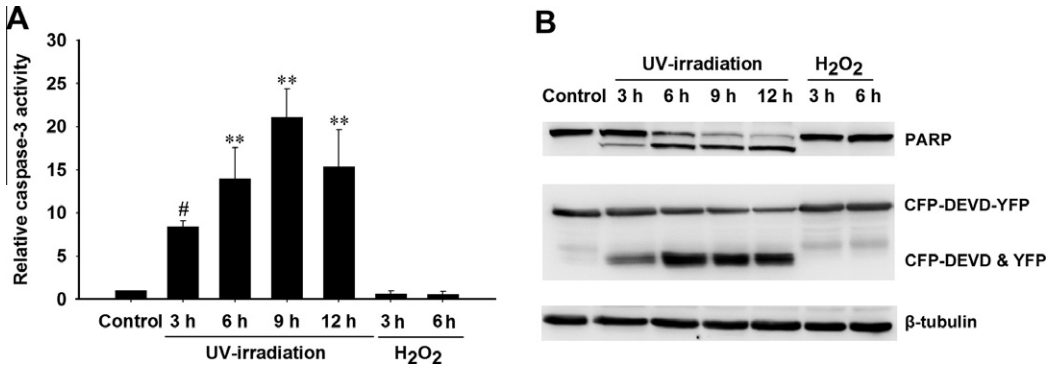

Fig. 2. Confirmation of caspase-3 activation during apoptosis. (A) *In vitro* caspase-3 activity assay and (B) Western blot analysis for PARP and biosensor C3 cleavage after cells were treated with apoptotic inducer 3 min UV-irradiation or necrotic inducer H_2O_2 (5 mM) followed by different periods of incubation. Data shown are means ± SD. $^{**}p < 0.01$, $^{\#}p < 0.001$ vs. control group, respectively.

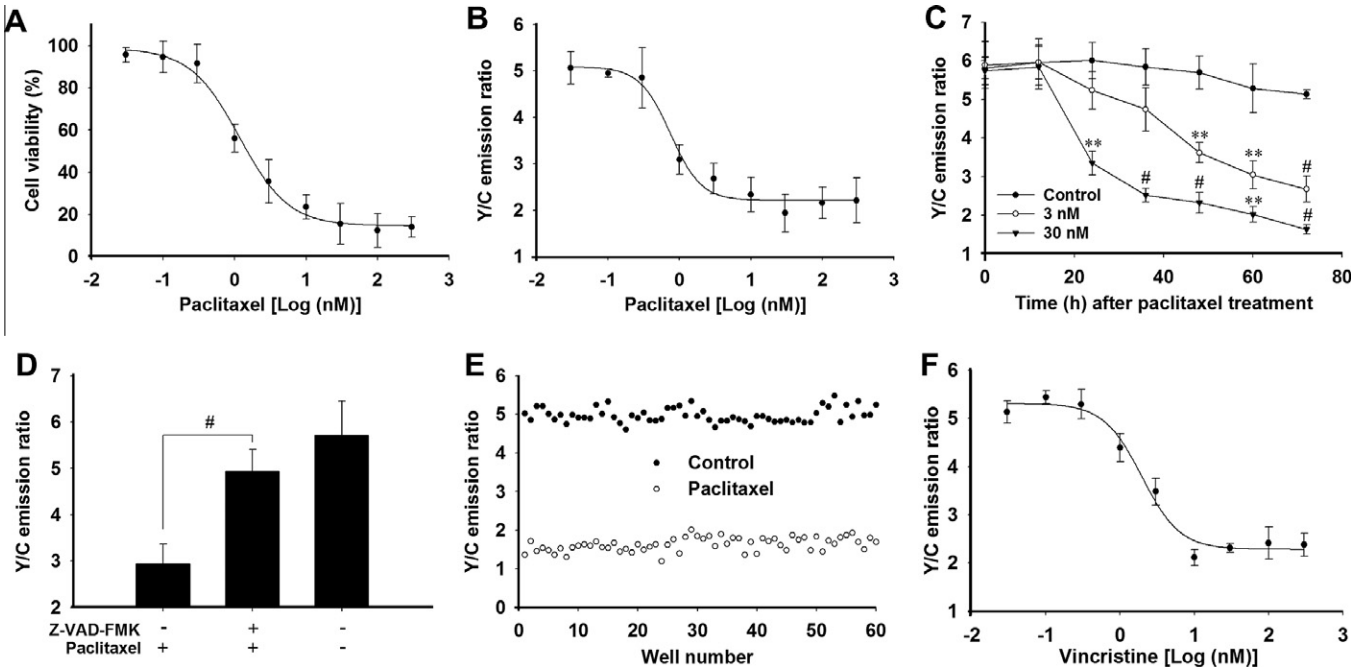

Fig. 3. Characterization of the effects of paclitaxel and vincristine on Y/C emission ratio in HUVEC-C3 cells. (A) The dose-dependent cytotoxic effect of paclitaxel after 72 h treatment measured by MTT assay. (B) The dose-dependent reductive effect of paclitaxel on Y/C emission ratio could be observed after 72 h treatment. (C) The temporal Y/C emission ratio reduction by 3 or 30 nM paclitaxel treatment. Data shown are means ± SD. $^{**}p < 0.01$, $^{\#}p < 0.001$ vs. control group, respectively. (D) Inhibitory effect of Z-VAD-FMK (40 μM) on Y/C emission ratio reduction induced by 36 h treatment of 30 nM paclitaxel. Data shown are means ± SD. $^{\#}p < 0.001$ vs. Z-VAD-FMK untreated cells. (E) Z' factor calculation by measuring the Y/C emission ratio value of positive treatment (30 nM paclitaxel treated for 72 h) and no treatment control. (F) The dose-dependent reductive effect of vincristine on Y/C emission ratio could be observed after 72 h treatment.

3.5. Application of HUVEC-C3 for VDA screening

Besides paclitaxel, we further tested this high-throughput screening platform with one of the VDA candidates, vincristine, though its high toxicity limited its clinical usage [12]. We measured the Y/C emission ratio changes after 72 h incubation of different concentrations of vincristine (Sigma–Aldrich). The concentration of vincristine at which it can reduce 50% of FRET effect was calculated to be 2.29 ± 0.09 nM from the dose–response curve (Fig. 3F). The CC_{50} value of vincristine determined by the MTT assay was 3.06 ± 0.04 nM. This result further validates the credential of using HUVEC-C3 cells to screen for compounds that can induce endothelial cell apoptosis as potential VDA candidates, and this method is more sensitive than the conventional cytotoxicity assay.

Then we utilized this system to screen for new VDA hits from our in-house herbal compounds library. Finally, the herbal compound dioscin showed good apoptotic activity against HUVEC-C3 cells. Dioscin (5 and 10 μM) could dramatically reduce the Y/C emission ratio (Fig. 4A). We also used the *in vitro* caspase-3 activity assay and Western blot analysis for PARP cleavage to confirm the apoptosis inducing ability of dioscin. After 24 h incubation of 10 μM of dioscin, the significant increase of caspase-3 activity (Fig. 4B) and PARP cleavage (Fig. 4C) were detected. We further used a cell-permeable DNA dye, Hoechst 33342 (100 ng/ml, Invitrogen) to stain the DNA in cells. As shown in Fig. 4D, dioscin (10 μM) treated for 18 h caused chromosome condensation and DNA fragmentation, which indicated cell apoptosis. The CC_{50} for dioscin was calculated to be 2.18 ± 0.56 μM by the MTT assay. Therefore dioscin could be considered as a potential VDA candidate.

4. Discussion

Endothelial cell apoptosis plays a critical role in pathology for many diseases. Therefore, understanding the regulation of endo-

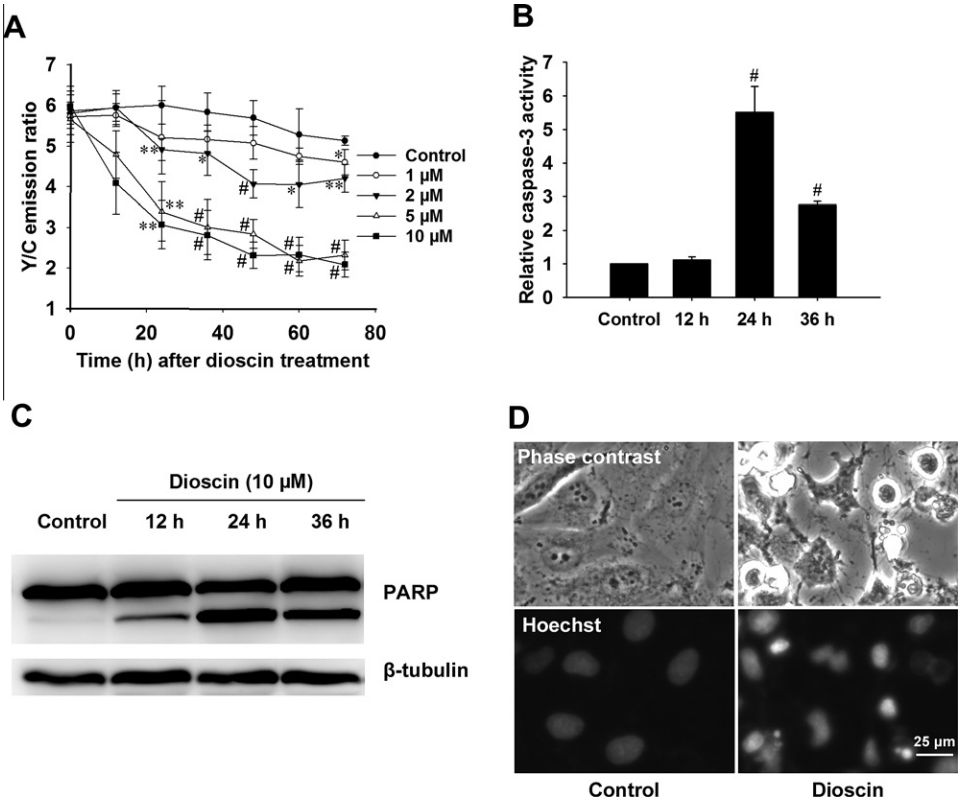

Fig. 4. Application of HUVEC-C3 for assessing the vascular disrupting effect of a herbal compound dioscin. (A) Dioscin reduced Y/C emission ratio in HUVEC-C3 cells in a dose and time dependent manner. (B) *In vitro* caspase-3 activity was enhanced after 10 μM dioscin treatment. (C) Western blot analysis for PARP cleavage after 10 μM dioscin treatment. (D) HUVEC-C3 cells were treated with 10 μM dioscin for 18 h and stained with a DNA dye Hoechst 33342. The fluorescent images showed that dioscin caused chromosome condensation and DNA fragmentation. Data shown are means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.001$ vs. control group, respectively. Scale bar = 25 μm.

thelial cell apoptosis, together with the goal of intervening in this process, has become a current research focus. A sensitive endothelial cell apoptosis assay using a FRET-based biosensor which can detect caspase-3 activity was firstly reported in this study.

Compared with the MTT assay which cannot distinguish cell apoptosis and necrosis, this assay can specifically detect caspase-3 dependent apoptotic cell death. Moreover, this cell-based assay enables the monitoring of caspase-3 activation at a single cell level and in real time in live endothelial cells. In order to overcome the drawback of time-consumption and labor intensity for the live imaging analysis of FRET effect, we developed a high-throughput method which evaluated the FRET changes by measuring the Y/C emission ratio using a fluorescent plate reader. The Z' factor which defines the feasibility of any high-throughput assay [11], was found to be above 0.5 for our present assay, thus indicating the reliability of this method.

Cell-based assays which provide insight into complex signal transduction pathways are powerful tools for drug discovery. We applied this assay for screening the vascular disrupting agents (VDA) which is used to destroy the existing blood vessels to deprive tumor blood supply to cancer cells within a solid tumor [13]. At present, the identified VDA can be classified into two groups based on their mechanisms of action. The first class is flavone acetic acid (FAA) derivatives which directly and indirectly disrupt tumor vasculature by inducing tumor endothelial cell apoptosis. The second class is tubulin-binding agents which induce rapid microtubule de-polymerization, leading to the disruption of microtubule structures. The resulting microtubule re-arrangement induces rapid endothelial cell round-up and increases vascular permeability followed by blood supply inhibition [13]. There are several screening methods for the identification of novel VDA, such as endothelial tube disruption, endothelial cell permeability, endothelial cell adhesion (rounding up), cell viability and apoptosis assay. For the second class of VDA (i.e. tubulin-binding agents) specifically, the *in vitro* tubulin binding or assembly assay is often used to characterize their effects. Though these agents will be used in clinic under a dose well below its cytotoxic effects, it is not a surprise to see that these drugs such as combretastatin A-4 [14], combretastatin A-4-phosphate [15] and AVE8062 [16] induce endothelial cell apoptosis for the reason of causing mitotic arrest. Thus our present selective endothelial cell apoptosis assay should be quite useful for novel VDA discovery.

HUVEC is the frequently used endothelial cell. Considering the engineered cell line stability, we used the immortalized HUVEC cell line in this study. This cell line shares many characteristics with primary endothelial cells including *in vitro* capillary formation and proliferative and differentiative response to growth factors [17]. Besides VDA screening, we also suggest that our reported HUVEC-C3 cells may be further utilized to optimize several currently used *in vitro* endothelial cell-based assays. For example, apoptotic inducers such as oxidized low-density lipoprotein (ox-LDL) [18] and high glucose [19] are believed as the triggering molecules that cause vascular injury, the related endothelial cell based apoptosis assays are commonly used to evaluate the effects of endothelium protective drugs. For this purpose, our developed cell-based assay may be adapted for screening the compounds which could inhibit endothelial cell apoptosis induced by a designated stimulus. Endothelial cells are also used to develop the *in vitro* angiogenesis models such as capillary formation assay. In some co-culture system-based tubule formation assays [20,21], our fluorescent HUVEC-C3 cells have the advantage to be distinguished from other co-cultured cells.

In summary, we developed an engineered endothelial cell line expressing FRET-based biosensor which can detect cell apoptosis. We optimized this assay into a high-throughput method in 96-well plates, and this system was validated by using apoptotic inducers including UV-irradiation, paclitaxel and vincristine. By the end, this assay was applied to screening VDA from an in-house compounds library. The identified VDA candidate dioscin was further characterized and confirmed in the endothelial cells. Therefore, this FRET-based endothelial cell apoptosis assay will be useful for evaluating endothelial cell apoptosis and screening VDA.

Acknowledgments

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