Original Contribution

SONODYNAMIC EFFECT OF AN ANTI-INFLAMMATORY AGENT – EMODIN ON MACROPHAGES

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Abstract—Emodin has been used as an anti-inflammatory agent and inflammation is a crucial feature of atherosclerosis. Here, we investigated the sonodynamic effect of emodin on macrophages, the pivotal inflammatory cells in atherosclerotic plaque. THP-1 derived macrophages were cultured with emodin and exposed to ultrasound. Six hours later, unlike the cells treated for 5 and 10 min, the viability of cells treated for 15 min decreased significantly and the cells showed typical apoptotic chromatin fragmentation. The percentage of apoptotic and necrotic cells in the sonodynamic therapy (SDT) group was higher than that in the ultrasound group. Two hours after treatment for 15 min, the cytoskeleton lost its original features as the filaments dispersed and the cytoskeletal proteins aggregated. The percentage of cells with disturbed cytoskeletal filaments in the SDT group was higher than that in the ultrasound group. These results suggest emodin has a sonodynamic effect on macrophages and might be used as a novel sonosensitizer for SDT for atherosclerosis. (E-mail: dzk@psu.edu and yetian@ems.hrbmu.edu.cn)

Key Words: Atherosclerosis, Inflammation, Macrophage, Emodin, Sonodynamic therapy.

INTRODUCTION

Atherosclerosis (AS) is a complex disease with several possible contributing factors. Inflammation is implicated in the pathogenesis of AS, especially in vulnerable plaque that triggers the onset of acute cardiovascular events (Hermus et al. 2010; Libby and Aikawa 2002). Photodynamic therapy (PDT) could induce the regression of atherosclerotic plaque (Waksman et al. 2008). The mechanism involves the effect of reducing the infiltration of macrophages, which are the metabolically active inflammatory cells in atherosclerotic plaque (Liu and Hamblin 2005). However, the application of PDT is limited to superficial lesions because of its definite penetration. Unlike PDT, sonodynamic therapy (SDT) can penetrate deeply into tissues (Tachibana et al. 2008).

The sonosensitizer is crucial for effective SDT. Most sonosensitizers come from photosensitizers (Wang et al. 2010c; Dai et al. 2009), which makes them liable to cause photodermatitis and they are not generally used in clinical practice. To avoid photodermatitis, developing a new sonosensitizer that can be widely used is necessary. Emodin from rhubarb, a natural herb, is widely used as a laxative and has other versatile biologic properties, such as anti-inflammation, anti-proliferation and anti-carcinoma (Liu et al. 2010; Chen et al. 2009; Lin et al. 2009; Hu et al. 2009; Cai et al. 2008). Emodin was found to stabilize atherosclerotic plaque in fat-fed apolipoprotein E-deficient mice, probably through its anti-inflammatory property (Zhou et al. 2008). Emodin is a naturally occurring and structurally related anthraquinone, which is a well-known photosensitizer (Buytaert et al. 2006). However, whether emodin can induce apoptosis or necrosis of human macrophages under ultrasound exposure is unknown.
We hypothesized that emodin could induce apoptosis or necrosis of macrophages under ultrasound exposure. In this study, we attempted to combine emodin with low-intensity ultrasound exposure to determine whether emodin can mediate sonodynamic therapy for macrophages.

MATERIALS AND METHODS

Reagents
Emodin was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The reagent is a commercial product of analytical grade with purity above 98% and it was dissolved in 100% dimethyl sulfoxide (DMSO) and stored at −20°C in the dark. The stock solutions were diluted 10- to 103-fold for the experiments. The final concentration of DMSO in cells was less than 0.1%. Roswell Park Memorial Institute (RPMI) medium (1640) and fetal bovine serum were obtained from Hyclone Chemical Co. (Thermofisher Biotechnology, Beijing, China). Benzylpenicillin-streptomycin was obtained from Beyotime Biotechnology (Jiangsu, China). Phorbol-12-myristate-13-acetate (PMA) was obtained from EMD Biosciences, Inc. (La Jolla, CA, USA). Hoechst 33342 and propidium iodide (PI) were purchased from Sigma Chemical Co., Ltd. (Santa Clara, CA, USA). A rabbit polyclonal antibody against vimentin (ab8545) was obtained from Abcam, Ltd. (Hong Kong, China). A rabbit polyclonal antibody against β-tubulin (H-235) and a goat polyclonal antibody against α-actin (C-11) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other drugs and chemicals used for this study were purchased from Sigma Chemical Co., Ltd.

Measurements of absorption and fluorescence spectra of emodin
The absorption spectrum of emodin was measured with a spectrophotometer (USB 2000; Ocean Optics Incorporated, Dunedin, FL, USA) under a deuterium lamp and a 40 W wolfram lamp. The fluorescence spectrum of emodin was measured with a spectrophotometer at a wavelength of 405 nm.

Cells culture
THP-1 cells (ATCC, Rockefeller, MD, USA) were cultured in RPMI Medium (1640) supplemented with 10% fetal bovine serum and 50 × 10^−3 g/L benzylpenicillin-streptomycin. Cells were maintained at 37°C with 5% CO2/95% air in a humidified incubator and they were harvested for passage when they reached confluence. For experiments, cells were seeded in microculture dishes (Costar; Corning Incorporated, Corning, NY, USA) in their usual medium plus PMA at a concentration of 10 × 10^−3 g/L for 72 h (Osto et al., 2008; Ning et al. 2009). The medium was removed and replaced with fresh medium without PMA.

Detection of intracellular uptake of emodin
Cells were seeded in 35-mm Petri dishes with 1 × 10^5 cells/mL. Emodin was added at a final concentration of 15 × 10^−3 g/L. After 2 h, the cells were washed twice with phosphate buffered saline (PBS) and the uptake of emodin by the cells was examined with a fluorescence microscope (IX71; Olympus, Tokyo, Japan) using a filter with an excitation wavelength of 420–480 nm and an emission wavelength of 480–550 nm.

Ultrasound exposure system
The ultrasound exposure system is shown in Figure 1 (838A-H-O-S ultrasonic device; Sheng Xiang Technology, Shenzhen, China). The ultrasound transducer, with a diameter of 38 mm, was submerged in a container filled with degassed water. The overall resonant frequency of the transducer was 0.86 MHz. The reading output power intensity from the amplifier was 2 W/cm². The target depth of the ultrasound was 10 cm below the transducer surface. The output acoustic pressure was measured in degassed water 10 cm from the transducer surface. The output intensity was 0.44 W/cm². For all experiments, degassed water was used as the ultrasonic medium. The solution inside the Petri dishes was buffered from overheating by the water around the Petri dishes, and the temperature was set to room temperature (23–25°C). During the sonication procedure, the temperature of the solution inside the Petri dishes did not rise more than 0.1°C, as measured with a thermometer.

Sonodynamic therapy
MTT assay. Cells (1 × 10^5 cells/mL) were seeded in 35-mm Petri dishes. Cells were incubated with emodin at a concentration of 15×10^−3 g/L for 2 h in the dark. They were then exposed to pulse ultrasound for 5–15 min. Control Petri dishes were sham-exposed to ultrasound. After SDT, each Petri dish was incubated for 6 h before

Fig. 1. The ultrasound exposure system.
the MTT assay was performed. Mitochondrial function was expressed as a percentage of viable treated cells relative to untreated control cells (without ultrasound and drug). All experiments were repeated three times independently.

**Hoechst PI.** Petri dishes were seeded with $1 \times 10^4$ cells/mL. The SDT experiments included an emodin concentration of $15 \times 10^{-3}$ g/L and ultrasound exposure for 15 min. Six hours later, the cell monolayer was stained with Hoechst-PI nuclear dye. The cell monolayer was washed twice with PBS, then examined under a fluorescence microscope using a filter with an excitation wavelength of 330–385 nm and an emission wavelength of 420–480 nm. The percentages of apoptotic and necrotic cells were calculated from the total cell numbers (total cells = alive + apoptotic + necrotic cells). All cells from 10 random microscopic fields at ×40 magnification were counted. Experiments were repeated three times independently.

**Immunofluorescence staining.** The SDT experiments included an emodin concentration of $15 \times 10^{-3}$ g/L and ultrasound exposure for 15 min. Two hours after SDT, the cells were fixed with paraformaldehyde (PFA). The cells were perforated with a detergent such as Triton X-100 to allow exposure of the antibodies to the structures inside the cells. To avoid non-specific binding of the second antibody, the cells were blocked with 1% BSA at room temperature for 1 h. Primary antibodies were added at $37^\circ$C for 1 h. Then, the secondary antibody, which was conjugated with fluorescein isothiocyanate (FITC), was added at $37^\circ$C for 2 h. 4',6-diamidino-2-phenylindole (DAPI) was added at room temperature for 2 min. The cell monolayer was washed twice with PBS and then examined under a fluorescence microscope using a filter with an excitation wavelength of 330–385 nm and an emission wavelength of 420–480 nm and one with an excitation wavelength of 420–480 nm and an emission wavelength of 480–550 nm. The status of cytoskeletal protein polymerization was calculated by randomly choosing at least 10 microscopic fields at ×40 and counting cells in the field as either having cytoskeletal filaments that were intact or disturbed. The percentage of cells with disturbed protein filaments was expressed as the number of cells with disturbed cytoskeletal filaments/the total number of cells.

**Statistical analysis**

All values were expressed as the means ± standard deviation. Dunnett-T and SNK tests were used to assess the effects of various emodin concentrations on cell viability without ultrasound exposure. The LSD and SNK tests were used to assess the effects of sonoactivated emodin on cell viability: $p$ values $< 0.05$ were considered significant.

**RESULTS**

Figure 2 shows the structure, absorption spectrum and emission spectrum of emodin. Panel A: the structure of emodin; panel B: the absorption spectrum of emodin under a wolfram lamp; panel C: the fluorescence emission spectrum of emodin at 405 nm.

![Fig. 2. The structure and spectrum of emodin. Panel A: the structure of emodin; panel B: the absorption spectrum of emodin under a wolfram lamp; panel C: the fluorescence emission spectrum of emodin at 405 nm.](image-url)
emission spectrum of emodin at 405 nm. The data show that the absorption wavelength of emodin is less than 500 nm and the fluorescence emission wavelengths of emodin range from 480 nm to 700 nm.

Figure 3 shows the distribution of emodin inside macrophages. Panel A: emodin fluorescence in unfixed macrophages; panel B: nuclei of the same cells after staining with Hoechst 33342 nuclear dye; panel C: a merged image of A and B. Emodin appears to be distributed in cytoplasm.

As shown in Figure 4, cell viability decreased significantly from 90 ± 9% (10 min) to 54 ± 5% (15 min) in cells treated with emodin-SDT. Cell viability decreased from 93 ± 5% (10 min) to 72 ± 9% (15 min) in cells treated with ultrasound alone. Cell viability decreased more significantly in cells treated with emodin-SDT for 15 min than ultrasound alone (54 ± 5% vs. 72 ± 9%, P < 0.01). Cell viability was not significantly affected in cells treated for 5 min and 10 min (p > 0.05). Treatment with emodin alone did not affect cell viability compared with the control (p > 0.05). DMSO at a concentration of 0.1% showed no effect on cell viability under ultrasound (data not shown).

Figure 5 shows the apoptosis and necrosis of cells 6 h after emodin-SDT. The controls and cells treated with emodin alone showed uniform blue fluorescence (Fig. 5A1 and A2); apoptotic cells were seen as bright blue fluorescence spots and necrotic nuclei were identified by the presence of staining with PI, which was evident as pink fluorescence (Fig. 5A3 and A4). The percentages of apoptotic cells in the ultrasound group and the SDT group were higher than that of the control (26 ± 6% vs. 4 ± 3%, p < 0.01; 32 ± 6% vs. 4 ± 3%, p < 0.01, respectively). The percentage of apoptotic cells in the SDT group was higher than that in the ultrasound group (32 ± 6% vs. 26 ± 6%, p < 0.05). The percentages of necrotic cells in the ultrasound group and the SDT group were higher than that of the control (5 ± 2% vs. 2 ± 1%, p < 0.05; 17 ± 5% vs. 2 ± 1%, P < 0.01, respectively). The percentage of necrotic cells in the SDT group was higher than that in the ultrasound group (17 ± 5% vs. 5 ± 2%, p < 0.01). There was no discernible difference in the percentage of apoptotic and necrotic cells between the emodin treated group and the controls.

Figure 6 shows morphologic changes of the cytoskeleton 2 h after emodin-SDT. Cells were fixed and incubated with α-actin, β-tubulin and vimentin antibodies to stain cytoskeletal protein (green fluorescence) and with DAPI to stain nuclei (blue fluorescence). Control cells showed a regular cytoskeletal network seen as green fluorescence, and nuclei showed uniform blue fluorescence (Fig. 6A-a1, b1 and c1). There were no obvious morphologic changes of the cytoskeleton in cells treated with emodin alone (Fig. 6A-a2, b2 and c2). The fluorescence signal of cytoskeletal protein was slightly attenuated 2 h after ultrasound exposure in some cells, as shown in Figure 6A-a3, b3 and c3 (indicated with arrowheads). In the case of cells treated with emodin-SDT, α-actin, β-tubulin and vimentin filaments dispersed and the proteins aggregated. The cytoskeleton lost its original features, as shown in Figure 6A-a4, b4 and c4 (indicated with arrows). The percentages of cells with disturbed cytoskeletal filaments in the ultrasound group and the SDT group were higher than that in the control (α-actin: 29 ± 5% vs. 3 ± 2%, p < 0.01; 45 ± 4% vs. 3 ± 2%, p < 0.01. β-tubulin: 29 ± 5% vs. 3 ± 2%, p < 0.01; 43 ± 9% vs. 3 ± 2%, p < 0.01. Vimentin: 29 ± 8% vs. 3 ± 1%, P < 0.01; 45 ± 7% vs. 3 ± 1%, p < 0.01, respectively). The percentage of cells with disturbed cytoskeletal filaments...
in the SDT group was higher than that in the ultrasound group (α-actin: 45 ± 4% vs. 29 ± 5%, p < 0.01; β-tubulin: 43 ± 9% vs. 29 ± 5%, p < 0.01; vimentin: 45 ± 7% vs. 29 ± 8%, p < 0.01). There was no difference between the emodin treated group and the controls.

**DISCUSSION**

SDT is based on energy transition to generate reactive oxygen species (ROS) (Rosenthal et al. 2004), which promote damage to biologic molecules including lipids,
proteins and DNA. Conjugated macro-π bonds are required to accomplish this energy transition. The more conjugated macro-π bonds inside sensitizer structures, the longer the absorption wavelength of the sensitizers. The number of macro-π bonds inside emodin (Fig. 2A) is less than that of hematoporphyrin and its derivatives (HPDs), so the absorption wavelength of emodin is shorter than that of HPDs. Through measurement of the absorption spectrum, it was shown that emodin absorbed light at wavelengths less than 500 nm (Fig. 2B and C), shorter than the optical window, that is to say, the minimum in the wavelength region 600–1000 nm enough to fulfill penetration into tissues. To resolve the issue of tissue penetration, ultrasound was used to activate emodin.

During SDT to atherosclerosis, the accumulation of the sonosensitizers at the atherosclerotic lesion is important. The sensitizer targets and accumulates in metabolically active inflammatory cells such as macrophages in the atherosclerotic plaque (Kereiakes et al. 2003; Bialy et al. 2003). In this study, the uptake of emodin by macrophages was detected. The accumulation of emodin in macrophages increased with concentration; however, drug cytotoxicity became obvious gradually along with the increase in concentration (data not shown). Therefore, the definition of a proper drug concentration is necessary. The results indicated that emodin concentration over $20 \times 10^{-3}$ g/L would kill cells but emodin concentrations below $5 \times 10^{-3}$ g/L...
did not exhibit intracellular drug fluorescence (data not shown). Through detection of emodin fluorescence and the same cell nuclei stained with Hoechst 33342, it was shown that emodin was distributed in the cytoplasm (Fig. 3). Liposoluble sensitizers likely enter cells through LDL-R (Kereiakes et al. 2003; Bialy et al. 2003) and because emodin is liposoluble, it probably enters the macrophages through this receptor and then translocates to the mitochondria to produce a marked effect.

Notable cell death was observed after emodin-SDT. Cell viability decreased gradually as the amount of ultrasound exposure increased. The cell survival rate in the emodin-SDT group was much lower than that in the ultrasound only group under the same exposure conditions (Fig. 4). The results indicated that emodin-SDT could effectively kill macrophages in vitro. Moreover, cell viability in the emodin-SDT group was not altered when cells contained only intracellular emodin (data not shown). Cell viability measured by MTT assay reflected dysfunction of the mitochondria. Mitochondrial dysfunction may result from the damage to the mitochondrial structure; it did not assess the late, irreversible changes that would indicate the mode of cell death. The Hoechst-PI assay repaired that deficiency. The nuclei of live cells presented uniform blue fluorescence because of resistance to the fluorescence dye (Fig. 5A1 and A2). Apoptotic nuclei presented bright blue fluorescent spots accompanied by nuclear deformation while necrotic nuclei presented pink fluorescence (Fig. 5A3 and A4). In this study, ultrasound exposure alone could induce cell death, which became obvious when the amount of sonication was augmented. This effect was highly enhanced when emodin was added to the cells. The percentage of both apoptotic and necrotic cells increased. There was a synergistic relationship between emodin and ultrasound. Therefore, emodin may be a promising natural sonosensitizer when used at the proper concentration and combined with the appropriate amount of sonication. Hematoporphyrin-SDT induced apoptosis of tumor cells through a mechanism that involved the mitochondria-caspase signaling pathway (Dai et al. 2009; Tang et al. 2010). The mechanism of macrophage apoptosis induced by emodin-SDT may also involve activation of the mitochondria-caspase signaling pathway.

Oxidative stress induced by PDT and SDT can affect several types of biomacromolecules including proteins, lipids and DNA (Davies 2003). In a number of papers, deleterious effects of PDT on the cytoskeleton have been documented (Sakharov et al. 2003; Panzarini et al. 2009; Jung et al. 2009; Casas et al. 2008). By a similar mechanism, SDT can also affect cytoskeletal proteins. Some papers showed that cytoskeleton might represent an important target for SDT (Zhao et al. 2009; Wang et al. 2010a). Cytoplasm skeletal proteins mainly consist of microfilaments, microtubules and intermediate filaments. Vimentin is a kind of middle fiber existing in cells. Its cleavage happens during early apoptosis. In this article, α-actin, β-tubulin and vimentin were observed. The fluorescence signal of cytoskeletal proteins in the cells treated with ultrasound alone was partially attenuated and this attenuation was greatly enhanced by adding emodin. Cytoskeletal filaments were cleaved and formed clusters. The cytoskeleton deformed. No obvious deformation of the cytoskeleton was observed in controls or in cells treated with emodin alone (Fig. 6). It is possible that the disruption of the cytoskeleton is one of the causes of cell death induced by emodin-SDT.

Currently, many possible sonosensitizers have been investigated but few have been approved for clinical use. Rhubarb has been widely used in folk medicine. Emodin, from rhubarb, demonstrates many pharmacologic properties such as regulating cell proliferation and inflammatory cytokines. Moreover, emodin injection has been used in the clinical treatment of viral hepatitis. Emodin promotes atherosclerotic plaque stability in vivo (Zhou et al. 2008). The combination of emodin and ultrasound may further stabilize or reduce the atherosclerotic plaque. Therefore, emodin-SDT may be a useful and promising treatment for atherosclerosis. Whether emodin-SDT can induce atherosclerotic plaque regression will require further study in animal models.

CONCLUSIONS

Emodin induces the apoptosis and necrosis of macrophages under ultrasound exposure. The results imply emodin-SDT might be a potential treatment for atherosclerosis by reducing the infiltration of macrophages in atherosclerotic plaque.

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