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Berberine reduces both MMP-9 and EMMPRIN expression through prevention of p38 pathway activation in PMA-induced macrophages

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ABSTRACT

Background: Overproduction of MMPs (matrix metalloproteinases) and EMMPRIN (extracellular matrix metalloproteinase inducer) by monocytes/macrophages leads to atherosclerotic plaque rupture by degrading the extracellular matrix. Serum MMP-9 levels may therefore represent a novel marker of inflammation in patients with known coronary artery disease. The purpose of our study was to determine if berberine, a natural extract from *Rhizoma coptidis*, had any effect on the expression of MMP-9 and EMMPRIN in PMA-induced macrophages.

Methods: Human monocytic THP-1 cells were pretreated with berberine for 1 h, and then induced by PMA for 48 h. Total RNA and protein were collected for Real-time PCR and Western blot analysis, respectively. Culture supernatants were collected to determine MMP-9 activity.

Results: In the present study, we demonstrated that berberine inhibited the expression of MMP-9 and EMMPRIN at both the mRNA and protein levels in a dose-dependent manner in PMA-induced macrophages, and that it also reduced MMP-9 activity. Furthermore, berberine also suppressed p38 signaling pathway activation in PMA-induced macrophages.

Conclusions: The data indicate that berberine reduces MMP-9 and EMMPRIN expression by suppressing the activation of p38 pathway in PMA-induced macrophages. This suggests a potential role for berberine as a therapeutic aid for stabilizing atherosclerotic plaque.

1. Introduction

The stability of atherosclerotic plaque determines whether a patient will experience stable angina pectoris or a life-threatening acute coronary syndrome, including acute myocardial infarction (AMI) and unstable angina pectoris (UA) [1]. MMP-9 (matrix metalloproteinase-9) and EMMPRIN are highly expressed in advanced atherosclerotic plaque [2,3] and have been shown to contribute to plaque rupture through degradation of the extracellular matrix [4,5]. In addition, serum MMP-9 has been characterized as a novel marker of acute myocardial infarction [6].

Of particular note, the synthesis of MMP-9 appears to be regulated by EMMPRIN in a paracrine or autocrine manner [7–9]. EMMPRIN was originally found on the surface of many tumor cells; however, increasing evidence has shown that EMMPRIN is strongly up-regulated in atherosclerosis-related cells, such as monocyte/macrophages [2,7], and in coronary smooth muscle cells [10], in response to certain proatherogenic stimuli, such as oxLDL. Likewise, EMMPRIN was reported to be increased in activated platelets [11] and in atherosclerotic plaques [2,7]. Taken together, this evidence suggests that EMMPRIN plays a key regulatory role in the development of atherosclerosis.

Since EMMPRIN and MMP-9 can promote instability of atherosclerotic plaque, it stands to reason that substances that inhibit EMMPRIN and MMP-9 expression would be potential candidates as agents for ameliorating the development of atherosclerosis. In this context, the isoquinoline alkaloid berberine is of interest. Berberine can be isolated from many medicinal herbs, such as *Hydrastis canadensis* (goldenseal), *Cortex phellodendri* (Huangbai) and *Rhizoma coptidis* (Huanglian) [12]. When present as a major ingredient, it has been shown to have pharmacological effects on cancer [13] and bacterial diseases [14]. In the present study, we investigated the effects of berberine on the expression of MMP-9 and EMMPRIN in PMA-induced macrophages.

2. Materials and methods

2.1. Reagents

RPMI 1640 medium, fetal bovine serum (FBS) and penicillin/streptomycin (pen/strep, 10,000 U/ml each) were purchased from GIBCO Company. Phorbol 12-myristate 13-acetate (PMA) was obtained from Calbiochem (San Diego, CA). Gelatin, dimethyl sulfoxide, and berberine were acquired from Sigma-Aldrich (St Louis, MO). Trizol reagent for RNA isolation was purchased from Invitrogen. Omniscript Reverse

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Transcriptase for first-strand cDNA synthesis was obtained from Qiagen. Anti-MMP-9, anti-phospho-p38 and anti-p38 antibodies were provided by Cell Signaling Technology (Danvers, MA). Rabbit monoclonal antibody to β-actin (ab8227) was produced by Abcam (Cambridge, UK). Anti-EMMPRIN antibody was obtained from Zymed (S. San Francisco, CA) and FITC-conjugated anti-EMMPRIN was from BD Biosciences. All goat anti-rabbit secondary antibodies (Catalog no.A-21109) used in Western blotting were from Invitrogen (Carlsbad, CA, USA). All other chemicals used for Western blotting were of the highest commercial purity available.

2.2. Cell culture

Human monocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained at a density of 10^6/ml in RPMI 1640 medium containing 10% FBS, 10 mM HEPES (Sigma) and 1% pen/strep solution at 37 °C in a 5% CO_2 incubator. The cells were cultured in six-well plates for 48 h in the presence of 100 nM PMA, which allowed them to differentiate into adherent macrophages [15]. The cells were incubated with 0 to 50 μM berberine.

2.3. Determination of cell viability (MTT assay)

The MTT assay was used to assess the cytotoxicity of berberine on PMA-induced macrophages. After the indicated treatments, the cells were incubated with 0.5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Roche Applied Science) in a culture medium for an additional 4 h. Next, the blue formazan crystals of viable cells were dissolved in dindimethyl sulfoxide and measured spectrophotometrically at 570 nm.

2.4. RNA isolation, cDNA synthesis and Taqman Real-time PCR

Total RNA was extracted from PMA-induced macrophages using Trizol reagent according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed into cDNA using random primers. The resultant cDNA was amplified by TaqMan Real-time polymerase chain reaction (PCR). The PCR reaction was directly monitored by the ABI-7300 Sequence Detection System (USA). The primer sequences are listed in Table 1. The whole amplification course was initiated at 95 °C for 5 min, followed by 40 cycles of 94 °C for 25 s, 55 °C for 25 s and 72 °C for 40 s. All results were normalized against GAPDH. All Real-time PCRs were run in duplicate.

2.5. Protein isolation and Western blot analysis

Protein isolation and Western blot analysis of cell lysates were performed as previously described [16] except that membranes were probed with primary antibodies for rabbit anti-MMP-9, anti-EMMPRIN, anti-phospho-p38 and anti-p38 (diluted 1:1000 in TBST), or rabbit anti-actin (diluted 1:5000 in TBST), followed by incubation with goat anti-rabbit secondary antibody labeled with far-red-fluorescent Alexa Fluor 680 dye. All signals were detected by an Odyssey imaging system (Li-cor, USA). All results were normalized against β-actin.

2.6. Flow cytometry

Flow cytometry was performed as described with the use of FITC-conjugated anti-EMMPRIN according to the manufacturer's protocol. The cells were analyzed on a BD FACSCalibur flow cytometer.

2.7. Gelatin zymography

MMP activity was determined by gelatin zymography. Briefly, culture supernatants were collected, 8 μl aliquots of the culture supernatant were loaded onto a 10% polyacrylamide gel containing 1 mg/ml gelatin and electrophoresed, then the gels were washed twice with 2.5% Triton X-100 (37 °C, 15 min) and incubated for 11 h with developing buffer (10 mM Tris Base, 40 mM Tris–HCl, 200 mM NaCl, 10 mM $CaCl_2$, 0.02% Brij 35) at 37 °C. The gels were stained with 0.5% (w/v) Coomassie Blue R-250 for 2 h and then destained in the solution (50% methanol, 10% glacial acetic acid, 40% water).

Table 1
Primers for Real-time PCR.

Gene	Sequences	Size (bp)
MMP-9	5′-CAGACATCGTCATCCAGTTTG-3′	192
	5′-CGCCATCTGCGTTTCCAA-3′	
	5′-fam-CCGAGTTGGAACCACGACGCCCTTG -tamra-3′	
EMMPRIN	5′-CTACACATTGAGAACCTGAACAT-3′	187
	5′-TTCTCGTAGATGAAGATGATGGT-3′	
	5′-fam-CAGCACCAGCACCTCAGCCACGATG-tamra-3′	
GAPDH	5′-CCAGGTGGTCTCCTCTGACTT-3′	130
	5′-GTTGCTGTAGCCAAATTCGTTGT-3′	
	5′-fam-AACAGCGACACCCACTCCTCCACC-tamra-3′	

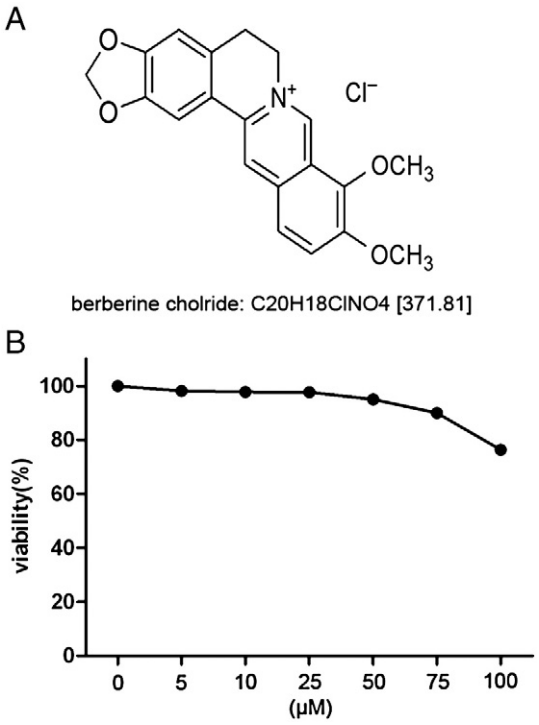

Fig. 1. Chemical structure of berberine and effects of berberine on the viability of PMA-induced macrophages. A: Chemical structure and molecular weight of berberine. B: Effects of berberine on cell viability. PMA-induced macrophages were treated with indicated concentrations of berberine (5–100 μM) and cell viability was assessed after 48 h using the MTT assay. Cells incubated in a medium without berberine were defined as control and considered 100% viable.

MMP activities were seen as clear bands against a blue background. An image of each gel was detected by an Odyssey imaging system (Li-cor, USA).

2.8. Statistical analysis

Data are expressed as mean ± S.D. Differences were compared by one-way ANOVA. A value of $p<0.05$ was considered statistically significant. All experiments were performed at least three times.

3. Results

3.1. Effects of berberine on cell viability

The structure of berberine used in this study is shown in Fig. 1A. The MTT assay was employed to evaluate the effect of berberine on the viability of PMA-induced macrophages. As shown in Fig. 1B, a concentration of berberine ranging from 5 to 75 μM caused no significant reduction (about 5–10%) in cell viability. A berberine dose under 75 μM was therefore considered to be non-cytotoxic and doses ranging from 5 to 50 μM were used in subsequent experiments.

3.2. Berberine reduced MMP-9 levels in PMA-induced macrophages at both mRNA and protein levels in a dose-dependent manner

To determine whether berberine has effects on MMP-9 at the mRNA and protein levels, THP-1 cells were pretreated with the indicated concentration of berberine for 1 h, followed by culture with PMA (100 nM) for 48 h. The amounts of MMP-9 mRNA were determined by Real-time PCR and the MMP-9 protein levels were quantified by Western blotting. Our results showed that berberine inhibited the expression of MMP-9, both at the protein and mRNA levels, in a dose-dependent manner (Fig. 2A, C). Thus, berberine reduced MMP-9 expression at both the transcription and translation level in PMA-induced macrophages.