In vitro effects of emodin on peritoneal macrophages that express membrane-bound CD14 protein in a rat model of severe acute pancreatitis/systemic inflammatory response syndrome

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Abstract. Emodin is the main active constituent of rhubarb and is often used in Chinese herbal medicine for the treatment of systemic inflammatory response syndrome (SIRS). The present study aimed to determine the *in vitro* effects of emodin on the expression of membrane-bound cluster of differentiation 14 (mCD14) protein in peritoneal macrophages (pMΦs). The severe acute pancreatitis (SAP)/SIRS model was established in Sprague-Dawley (SD) rats via retrograde injection of 1.5% sodium deoxycholate into the common biliopancreatic duct. The 40 SD rats were randomly divided into the sham-operated (n=10) group (SO) and the model group (n=30). After 24 h, pMΦs were harvested and the model group was randomly divided into three subgroups (n=10 per group), the 5 μg/ml emodin group (EMO), the 0.1 μmol/ml dexamethasone group (DEX) and the SIRS/SAP group (SI). Treatment agents were administered following macrophage adhesion for 24 h. Compared with that of the SO group, the SI group showed significantly increased pathological changes (P<0.01). Compared with that of the SO group, mCD14 expression in pMΦs was significantly decreased in the SI group (P<0.01). Additionally, compared with that of the SI group, mCD14 expression in pMΦs was significantly increased in the EMO group (P<0.01) and in the DEX group (P<0.01). Compared with that of the DEX group, mCD14 expression in pMΦs was significantly increased in the EMO group (25.60±2.79 vs. 20.87±1.99; P<0.01). The pathological changes observed in the pancreas of rats in the model groups were more severe than that of the SO group. Moreover, mCD14 expression levels in pMΦs were significantly decreased in the SI group. The pathological changes of each intervention group improved to various degrees, particularly in the EMO group.

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Abbreviations: mCD14, membrane-bound CD14 receptor; sCD14, soluble CD14 receptor; SO, sham-operated group; EMO, emodin group; DEX, dexamethasone group; SI, SIRS/SAP group; SAP, severe acute pancreatitis; SIRS, systemic inflammatory response syndrome; MODS, multiple organ dysfunction syndrome; MΦs, macrophages; pMΦs, peritoneal macrophages; PMN, polymorphonuclear neutrophil; LPS, lipopolysaccharide; LPB, lipopolysaccharide binding protein

Key words: emodin, severe acute pancreatitis, systemic inflammatory response syndrome, macrophage, membrane-bound cluster of differentiation 14 protein.

Introduction

Acute pancreatitis is currently one of the most intractable diseases in the surgical acute abdomen. Due to the high incidence rate and rapid progression, particularly for severe acute pancreatitis (SAP) with systemic inflammatory response syndrome (SIRS), the mortality rate of acute pancreatitis is high (1,2). SIRS is an early manifestation of multiple organ dysfunction syndrome (MODS) and multiple organ failure (MOF) (1,2).

It has been previously demonstrated that the polymorphonuclear neutrophil (PMN) life cycle is prolonged and apoptosis is delayed during infection, trauma and other stresses, which subsequently promotes inflammatory reactions that result in organ injury (3-8). Inflammatory reactions are worsened unless apoptotic PMNs are ameliorated. Therefore, delayed apoptosis of PMNs and the insufficient phagocytosis of apoptotic cells can increase inflammation. The majority of apoptotic cells *in vivo* are removed by macrophages (MΦs). MΦs identify, adhere and phagocytoze apoptotic PMNs to subsequently inhibit inflammatory reactions and promote inflammation resolution (9). Membrane-bound CD14 (mCD14) is one of the main receptors for MΦ recognition, inducing the phagocytosis of apoptotic neutrophils (10). In the present study, a model of SAP/SIRS was established in rats to investigate the *in vitro* effects of emodin compared with that of dexamethasone (DEX) on the expression levels of mCD14 protein in peritoneal MΦs (pMΦs).

Materials and methods

Animals. Healthy male Sprague-Dawley (SD) rats (weight, 220-250 g) were provided by the Laboratory Animal Center of Dalian Medical University (Dalian, China). Forty SD rats were randomly divided into the sham-operated group (SO) (n=10) and the model group (n=30). After 24 h, pMΦs were harvested and the model group was randomly divided into three groups (n=10 in each group); the 5 μg/ml emodin group (EMO), the 0.1 μmol/ml DEX and the SIR/SAP group (SI). The therapeutic agents were administered following pMΦ adhesion for 24 h. The study was approved by the Ethics Committee of the First Affiliated Hospital of Dalian Medical University (Dalian, Liaoning, China).

Equipment. High-speed refrigerated 5840R centrifuge (Eppendorf, Hamburg, Germany), flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) and an immunofluorescence microscope (CX31-32RFL; Olympus, Tokyo, Japan) were used in this study.

Reagents and drugs. RPMI-1640 medium, fetal bovine serum, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit, rabbit anti-CD14 polyclonal antibody and emodin were purchased from Sigma (St. Louis, MO, USA). Dextran T500 was purchased from Sigma.

SAP/SIRS model establishment. The SAP/SIRS rat model was established as described previously (11). Briefly, rats fasted for 12 h prior to surgery, but had access to water *ad libitum*. Rats were then anesthetized intraperitoneally with an injection of 10% chloral hydrate (Sigma) at a dose of 0.3 ml/100 g. To expose the duodenum, a midline laparotomy was performed. A 1 ml syringe needle was inserted through the contralateral intestinal wall of the duodenal papilla into the bile and pancreatic ducts, and clamped using a noninvasive bulldog clamp, followed by slow retrograde perfusion of 1.5% sodium deoxycholate (0.1 ml/100 mg) for 60 sec. The duodenal papilla was pinched to prevent back flow. The SO group were only subjected to a celiotomy.

Isolation, purification, culture and administration of pMΦs. Trypan blue staining was performed to detect the pMΦ survival rates and cell purity was >98 and >95% in the SO and model groups, respectively. The majority of cells showed the morphological features of MΦs. pMΦs from each group were seeded in 6-well culture plates and treated with 5 μg/ml emodin or 0.1 μmol/ml DEX. The SI and SO groups were untreated. Cells were then incubated at 37˚C with 5% CO_2 for 24 h.

Pathomorphological observation of pancreatic tissue. Pancreatic tissue sections, cut from the formalin-fixed, paraffin-embedded tissues, were routinely assessed with hematoxylin and eosin staining. The pathological scoring was performed according to the method of Kaiser *et al* (11).

Detection of mCD14 protein expression in pMΦs using immunofluorescence. Glass slides were placed into 24-well culture plates. PMΦ concentration was adjusted to $5x10^6$ cells/ml. After 30 min, cells in 2 ml RPMI-1640 containing serum were added to the culture plates and incubated at 37˚C with 5% CO_2 for 24 h. Emodin (5 μg/ml) and DEX (0.1 μmol/ml) were respectively added into separate wells, followed by incubation at 37˚C with 5% CO_2 overnight. Cells were harvested, washed with phosphate-buffered saline (PBS) three times, dried, fixed with 4% paraformaldehyde for 30 min and then washed again with PBS three times. One drop of non-immune animal serum was added to each slide and incubated at room temperature for 30 min. Additionally, 100 μl rabbit anti-CD14 polyclonal antibody (1:100; Sigma) was added to each slide and incubated at room temperature for 30 min in the dark. Slides were washed with PBS three times for 5 min and then dried. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (50 μl; Sigma) was added to each slide and incubated at room temperature for 30 min. Slides were washed with PBS three times for 5 min, dried and observed under a fluorescence microscope (Olympus). Positive reactions were detected under a fluorescence microscope as green fluorescent particles, indicating the presence of pMΦ mCD14 receptors.

Detection of mCD14 expression in pMΦs using flow cytometry. After 24 h of culture, cells were washed three times in pre-warmed Hank's solution (Beyotime, Shanghai, China) and then trypsinized with 0.25% trypsin at 37˚C for 5-6 min. When 90% of the adhered pMΦs appeared round and transparent as observed under an inverted microscope (Nikon Eclipse TS100; Nikon Corporation, Tokyo, Japan), digestion was terminated with 10-20 ml RPMI-1640 medium and cells were triturated. Cells were centrifuged at 111.8 x g for 10 min at 4˚C. The supernatant was discarded and cells were incubated with 2 μl rabbit anti-CD14 polyclonal antibody (Sigma) at room temperature for 1 h, washed with PBS three times, centrifuged at 1,000 r/min (R=10 cm) for 10 min at 4˚C, and incubated with 2 μl FITC-conjugated goat anti-rabbit antibody (Sigma) at room temperature for 15 min. Cells were then washed three times with PBS and centrifuged at 1,000 r/min for 10 min at 4˚C. Cells were resuspended in 1 ml PBS and mCD14 expression was determined by flow cytometry.

Statistical analysis. Data were analyzed using SPSS software, version 11.5 (SPSS, Inc., Chicago, IL, USA) and expressed as the mean ± standard deviation. Enumeration data were analyzed using the exact probability of a 4-fold table and measurement data with completely random analysis of variance. Paired comparison was performed using a q-test. $P<0.05$ was considered to indicate a statistically significant difference at an α level of 0.05.

Results

Clinical manifestations. Following injection of 1.5% sodium deoxycholate, rats exhibited rapid breathing and symptoms were aggravated with prolonged time. Rats also presented with cyanosis of the mucosa of the skin, unconsciousness and occasionally death (mortality rate was 20% in the model groups).

Gross observation. Immediately following injection of 1.5% sodium deoxycholate, the pancreatic gland presented with evident regional or diffuse hyperemia and edema, with increased pancreatic envelope tension. After 24 h, pancreatic

Table I. Comparison of the pathological changes in the SO and SI groups.

Group	No. of rats	Edema	Inflammation	Hemorrhage	Necrosis	P-value
SO	10	0.40±0.07	0	0	0	<0.01
SI	8	3.50±0.21	2.70±0.16	2.30±0.19	2.80±0.25	

SO, sham-operated group; SIRS/SAP, SI group. Values are expressed as the mean ± standard deviation.

hemorrhaging, necrosis and bloody ascites were observed in the surviving rats of the model groups. In addition to yellow saponaceous spots in the greater omentum and common bile duct, lung hyperemia, edema, bleeding in the lung, stomach edema, paralytic expansion, liver swelling and kidney augmentation were observed. However, in the SO group, mild edema of gastrointestinal mucosa and exudation in the abdominal cavity were observed.

Pathological changes in pancreatic tissues observed by light microscopy. The SO group exhibited clear pancreatic lobule structures. The model groups presented with necrosis in the pancreatic glandular parenchyma, bleeding, fatty degeneration, erythrocyte stasis, angiectasis and PMN infiltration into the tissue space and parenchyma. Compared with the SO group, the SI group showed significantly increased pathological changes (edema, 3.50±0.21 vs. 0.40±0.07; inflammation, 2.70±0.16 vs. 0; bleeding, 2.30±0.19 vs. 0 and necrosis, 2.80±0.25 vs. 0; P<0.01; Table I and Fig. 1).

mCD14 expression in pMΦs in each group. As detected by immunofluorescence, green fluorescent particles were observed in the SO, EMO, DEX and SI groups, indicating the presence of pMΦ mCD14 receptors (Fig. 2).

The mCD14 expression levels in pMΦs in each group detected by flow cytometry (mean ± standard deviation) are shown in Table II and Fig. 3. Compared with the SO group, the expression levels of mCD14 in pMΦs were significantly decreased in the SI group (28.55±2.53 vs. 14.76±2.84; P<0.01). Compared with the SI group, the expression levels of mCD14 in pMΦs were significantly increased in the EMO (25.60±2.79 vs. 14.76±2.84; P<0.01) and DEX (20.87±1.99 vs. 14.76±2.84; P<0.01) groups. Compared with the DEX group, mCD14 expression levels in pMΦs were significantly increased in the EMO group (25.60±2.79 vs. 20.87±1.99; P<0.01).

Table II. mCD14 expression levels in pMΦs in each group as detected by flow cytometry.

Groups	No. of rats	Expression rate (%)
SO	10	28.55±2.53
SI	8	14.76±2.84[a]
EMO	8	25.60±2.79[b]
DEX	8	20.87±1.99[b,c]

[a]P<0.01, vs. the SO group; [b]P<0.01, vs. the SI group; and [c]P<0.01, vs. the EMO group. SO, sham-operated group; SI, SIRS/SAP group; EMO, emodin group; DEX, dexamethasone group; pMΦ, peritoneal macrophage; mCD14, membrane-bound cluster of differentiation 14 receptor. Values are expressed as the mean ± standard deviation.

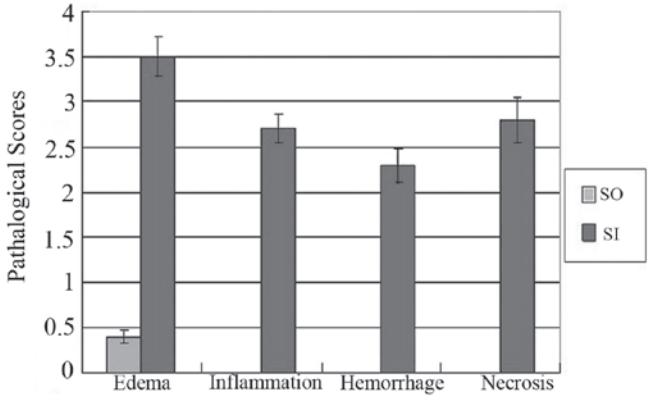

Figure 1. Comparison of the pathological changes in the SO and SI groups. Compared with the SO group, the pathological changes of the SI group were more severe according to the pathological scoring. SO, sham-operated group; SI, SIRS/SAP group.

Discussion

There are numerous receptors, which are involved in the recognition and phagocytosis of apoptotic cells, on the surface of phagocytes. The protein, CD14, is one of the five macrophage receptors located on the surface of phagocytes, which is involved in the recognition and phagocytosis of apoptotic cells. The expression of CD14 is tissue and cell specific. CD14 is mainly expressed on the surface of mononuclear MΦs and is weakly expressed on the surface of neutrophils and lymphocytes (12-15). The expression levels of CD14 are different from that of various types of mononuclear MΦs; the expression of CD14 is stronger on the surface of pMΦs and weaker on the surface of alveolar macrophages, Kupffer and microglia cells (16). Moreover, numerous factors can affect the expression levels of CD14 in the development process of monocytes to MΦs. There are two forms of CD14, termed mCD14 and soluble CD14 receptor (sCD14). sCD14 appears in the serum due to proteolytic cleavage and phospholipase D-induced mCD14 shedding from monocytes (10). mCD14 is one of the predominant receptors that MΦs recognize inducing the phagocytosis of apoptotic cells. CD14 is a receptor for the serum lipopolysaccharide (LPS) complex or LPS binding protein (LBP) and may be shed from the monocyte surface to inhibit the release of tumor necrosis factor-α (TNF-α) (17). In addition, Marchant *et al* (18) found that a low concentration

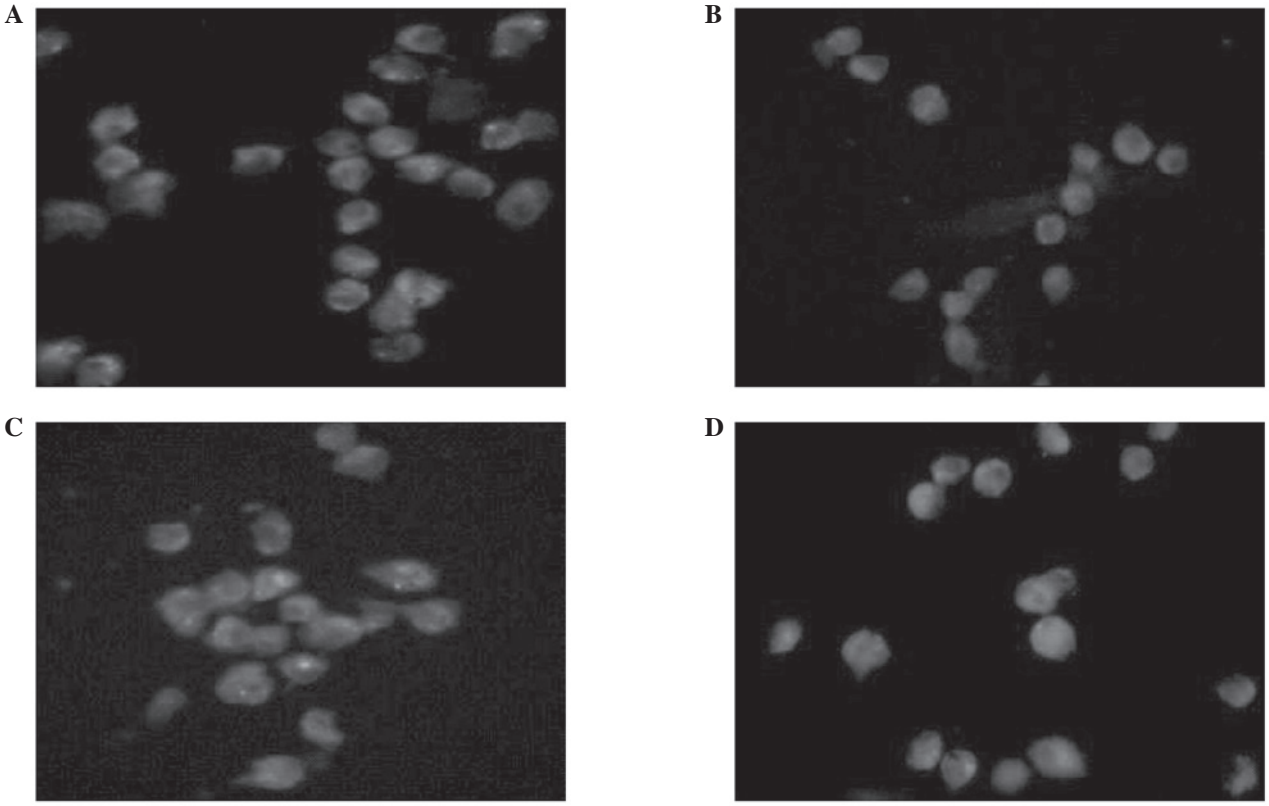

Figure 2. As detected by immunofluorescence, green fluorescent particles were found in the (A) SO, (B) SI, (C) EMO and (D) DEX groups, indicating the presence of pMΦ mCD14 receptors in each group (magnification, x100). SO, sham-operated group; SI, SIRS/SAP group; EMO, emodin group; DEX, dexamethasone group; pMΦ, peritoneal macrophage; mCD14, membrane-bound cluster of differentiation 14 receptor.

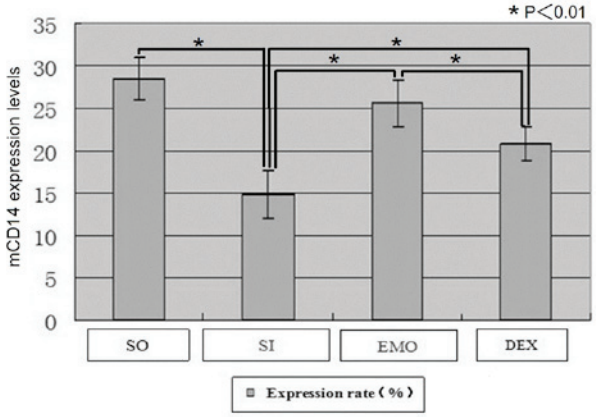

Figure 3. mCD14 expression levels in pMΦs in each group detected by flow cytometry. Compared with the SO group, mCD14 expression levels in pMΦs were significantly decreased in the model groups. Compared with the SO group, mCD14 expression levels in pMΦs were significantly decreased in the SI group (P<0.01). Compared with the SI group, mCD14 expression levels were significantly increased in the EMO (P<0.01) and DEX (P<0.01) groups. Compared with the DEX group, mCD14 expression levels in pMΦs were significantly increased in the EMO group (P<0.01). PMΦ, peritoneal macrophage; mCD14, membrane-bound cluster of differentiation 14 receptor; SO, sham-operated group; SI, SIRS/SAP group; EMO, emodin group; DEX, dexamethasone group.

of LPS increased the expression levels of monocyte mCD14. In the present study, mCD14 protein expression levels in pMΦs were significantly decreased following the induction of SAP/SIRS compared with that of the SO group. Therefore, it was speculated that SIRS induced intestinal mucosal barrier damage and bacterial translocation causes intestinal endotoxemia. After binding to LBP receptors on MΦs thereby forming LPS-LBP complexes, endotoxins could induce MΦs to synthesize and release a large quantity of TNF-α and other inflammatory mediators that trigger inflammatory reactions (16,19-22). Additionally, mCD14 may be shed from the monocyte surface accompanied by a decrease in the levels of mCD14.

Dahuang (Rhubarb), a Chinese herb, has been found to be clinically effective for the treatment of acute pancreatitis (23-26). Emodin is the main active component of Dahuang. Previous studies have shown that emodin affects bacteriostasis, catharsis, releases Oddi sphincter spasms, inhibits abnormal metabolism of vasoactive substances (eicosenoic acid), improves microcirculation and antagonizes coagulation and thrombus formation (23,27,28). The present study showed that emodin significantly increased mCD14 protein expression levels in pMΦs in rats with SAP/SIRS compared with that of the SI (P<0.01), which subsequently enhanced the identification and phagocytosis capacity of pMΦs and relieved inflammatory reactions.

In 1952, Stephensen *et al* (29) first reported the application of glucocorticoids to treat acute pancreatitis. However, the mechanisms underlying their effect have not been fully elucidated. The effects of glucocorticoids on inflammation via receptor mediation may trigger anti-inflammation. Glucocorticoids inhibit inflammatory exudation, leukocytic infiltration, inflammatory mediator production and release, as

well as improve microcirculation, alleviate endotoxemia and induce apoptosis of pancreatic acinar cells, thereby reducing the degree of pancreatic necrosis in SAP (30,31). A previous study demonstrated that pancreatic cell apoptosis occurs during acute pancreatitis (32). Animal studies have also indicated that DEX induces pancreatic cell apoptosis, stabilizes the internal environment and attenuates inflammation in pancreatic tissues (31,33). In the present study, it was demonstrated that DEX increased mCD14 expression levels in pMΦs.

In this study, it was demonstrated that compared with the SO group, mCD14 expression levels in pMΦs were significantly decreased in the model groups. *In vitro* emodin or DEX administration increased the expression levels of mCD14 in pMΦs. Notably, emodin exhibited more significant effects than DEX, suggesting that emodin and DEX may be used together due to their respective advantages.

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