In vitro effects of emodin on peritoneal macrophage intercellular adhesion molecule-3 in a rat model of severe acute pancreatitis/systemic inflammatory response syndrome

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Abstract. Rhubarb is often used in Chinese herbal medicine for the treatment of systemic inflammatory response syndrome (SIRS). Emodin is the main active constituent of rhubarb. This study was performed to investigate the *in vitro* effects of emodin and dexamethasone on peritoneal macrophage (pMΦ) phagocytosis and the expression of intercellular adhesion molecule-3 (ICAM-3). A total of 40 Sprague-Dawley (SD) rats were randomly divided into sham surgery (n=10) and model groups (n=30). After 24 h, pMΦs were harvested and the model group was randomly divided into three subgroups (n=10 rats/subgroup): the 5 μg/ml emodin, 0.1 μmol/ml dexamethasone and control groups. The drugs were administered following macrophage (MΦ) adhesion for 24 h. pMΦ phagocytosis was significantly increased in the emodin group compared to that in the control group. Moreover, pMΦ phagocytosis was significantly increased in the emodin group compared to that in the dexamethasone group. The expression of ICAM-3 was significantly increased in the emodin group compared to that in the control group. The expression of ICAM-3 was significantly increased in the emodin group compared to that in the dexamethasone group. The expression of ICAM-3 was significantly increased in the emodin and dexamethasone groups compared to that in the control group. pMΦ phagocytosis and ICAM-3 expression were significantly increased following emodin treatment compared to those in the control and dexamethasone groups, indicating that emodin may enhance pMΦ phagocytosis and apoptotic cell clearance by altering ICAM-3 expression.

Introduction

Acute pancreatitis is a common cause of clinical acute abdomen. Approximately 20-25% of patients with acute pancreatitis develop severe acute pancreatitis (SAP), which tends to be complicated by systemic inflammatory response syndrome (SIRS), with a high mortality rate >30% (1-3). SIRS is an early manifestation of multiple organ dysfunction syndrome and multiple organ failure.

It was previously reported that the polymorphonuclear neutrophil (PMN) life cycle is prolonged and apoptosis is delayed during infection, trauma and other types of stress, which promotes inflammatory reactions leading to organ injury (4-9). Inflammatory reactions may become more severe, unless apoptotic PMNs are cleared. Therefore, delayed PMN apoptosis and insufficient phagocytosis of apoptotic cells may enhance inflammation. The majority of apoptotic cells *in vivo* are cleared by macrophages (MΦs). MΦs identify, adhere to and phagocytize apoptotic PMNs to inhibit inflammatory reactions and promote inflammation absorption (10).

Intercellular adhesion molecule-3 (ICAM-3) is involved in cell adhesion and signal transduction (11,12). ICAM-3 is mainly expressed by leukocytes and highly expressed by lymphocytes, monocytes and neutrophilic granulocytes. ICAM-3 on apoptotic cells binds MΦ CD14 via bridging molecules to induce Ca^{2+} flow and phosphatidylserine externalization and promote the clearance of apoptotic cells (13).

The present study established a rat model of SAP/SIRS to investigate the *in vitro* effects of emodin (1,3,8-trihydroxy-6-methylanthraquinone; Fig. 1) compared to those of dexamethasone on peritoneal macrophage (pMΦ) ICAM-3 protein expression and phagocytosis.

Materials and methods

Animals. A total of 40 healthy male Sprague-Dawley (SD) rats, weighing 220-250 g, were provided by the Laboratory Animal Center of Dalian Medical University. The SD rats were randomly divided into sham surgery (n=10) and model

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Figure 1. Chemical structure of emodin.

trypsinized at 37˚C for 5-6 min. After 90% of the adhered MΦs were round and transparent, as observed under an inverted microscope, digestion was terminated by addition of 10-20 ml RPMI-1640, followed by trituration. The cells were centrifuged at 111.8 x g for 10 min at 4˚C. The supernatant was discarded and the cells were incubated with 1 μl anti-ICAM-3 antibody at room temperature for 1 h, washed with phosphate-buffered saline (PBS) three times, centrifuged at 111.8 x g (radius=10 cm) for 10 min at 4˚C and incubated with a FITC-conjugated goat anti-rabbit antibody at room temperature for 15 min. Subsequently, the cells were washed with PBS three times and centrifuged at 111.8 x g for 10 min at 4˚C. The cells were resuspended in 1 ml PBS and ICAM-3 expression was determined by flow cytometry.
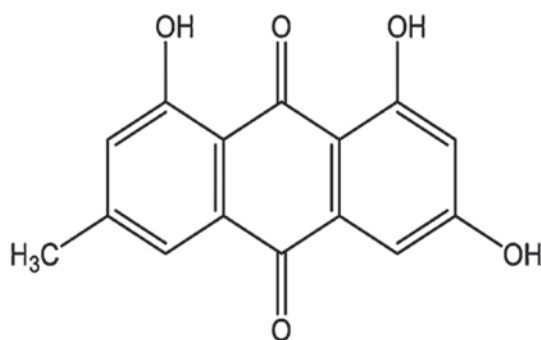
(SAP/SIRS) groups (n=30). pMΦs were harvested from the model group and the rats were randomly divided into three subgroups (n=10/subgroup): the emodin (5 μg/ml), dexamethasone (0.1 μmol/ml) and control groups. The drugs were administered following MΦ adhesion for 24 h.

Equipment. A high-speed refrigerated 5840R centrifuge was obtained from Eppendorf, Hamburg, Germany, a flow cytometer (FACSAsia) was purchased from BD Biosciences, Franklin Lakes, NJ, USA and an immunofluorescence microscope (CX31-32RFL) was purchased from Olympus Corporation, Tokyo, Japan.

Reagents and drugs. RPMI-1640 medium, fetal bovine serum (FBS), rabbit anti-ICAM-3 antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody and emodin and dianisidine were purchased from Sigma, St. Louis, MO, USA; Dextran T500 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

SAP/SIRS model establishment (14). The rats were fasted with free access to water for 12 h prior to surgery. The rats were then anesthetized with an intraperitoneal injection of 10% chloral hydrate at a dose of 0.3 ml/100 g. To expose the duodenum, a midline laparotomy was performed. An 1-ml syringe needle was inserted through the intestinal wall contralateral to the duodenal papilla into the bile and pancreatic ducts and clamped using a non-invasive 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 backflow. The sham-surgery group was only subjected to a celiotomy.

Isolation, purification, culture and administration of pMΦs. Trypan blue staining revealed that the pMΦ survival rate and purity were >98 and >95%, respectively. The majority of cells exhibited the morphological characteristics of MΦs. pMΦs from each group were seeded in 6-well culture plates and treated with 5 μg/ml emodin and 0.1 μmol/ml dexamethasone. The control and sham-surgery groups were untreated. The cells were then incubated at 37˚C with 5% CO_2 for 24 h.

Detection of pMΦ ICAM-3 expression using flow cytometry. After a 24-h culture, the cells were washed three times in pre-warmed Hanks' balanced salt solution and 0.25%

PMN isolation and culture. The PMNs were isolated according to the method of Percoll density gradient centrifugation. The cells were suspended in RPMI-1640 medium supplemented with 100 U/ml streptomycin, 100 U/ml penicillin and 10% heat-inactivated FBS. The cell density was adjusted to $5x10^6$ cells/ml. Trypan blue staining revealed that the PMN survival rate and purity were >95%.

Isolated PMNs were seeded at $5x10^6$ cells in 2 ml medium per well in 24-well culture plates and incubated at 37˚C in a humidified atmosphere with 5% CO_2 for 24 h. PMN apoptosis was observed and the cells were collected.

Determination of rate and index of pMΦ phagocytosis. PMNs (200 μl) were co-cultured with pMΦs (pMΦ:PMN = 1:5) at 37˚C with 5% CO_2 for 30 min. Non-phagocytized PMNs were washed with pre-cooled PBS, fixed with 4% paraformaldehyde overnight and mixed with equal quantities of 1.25 mg/ml dianisidine and 0.05% H_2O_2 for myeloperoxidase (MPO) staining. MPO-positive PMNs exhibited brown staining, whereas MΦs were negative. The positively stained pMΦs were considered to be those that phagocytized PMNs and were quantified by microscopy (magnification, x40). Three regions were randomly selected, 100 cells were quantified and the mean was calculated. The phagocytosis rate and phagocytic index were calculated to indicate phagocytosis ability as follows:

$$\text{Phagocytosis rate} = \frac{\text{Number of pM}\Phi \text{ phagocytizing PMN}}{100\,pM\Phi} \times 100\% \text{ (mean of three regions)}$$

$$\text{Phagocytic index} = \frac{\text{Number of phagocytized MPO}}{100\,pM\Phi} \text{ (mean of three regions)}$$

Detection of MΦ ICAM-3 protein expression using immunofluorescence. The glass slides were placed in 24-well culture plates and the MΦ concentration was adjusted to $5x10^6$ cells/ml. After 30 min, cells in 2 ml RPMI-1640 containing serum were added and incubated at 37˚C with 5% CO_2 for 24 h. Emodin (5 μg/ml) and dexamethasone (0.1 μmol/ml) were added to the emodin and dexamethasone groups, respectively, followed by incubation at 37˚C with 5% CO_2 overnight.

The cells were harvested, washed with PBS three times, dried, fixed with 4% paraformaldehyde for 30 min and washed with PBS another three times. One drop of non-immune animal serum was added to each slide and incubated at room temperature for 30 min.

One drop of primary antibody (dilution 1:100, 100 μl) was then added to each slide and incubated at room temperature for 30 min in the dark, washed with PBS for 3x5 min and dried.

FITC-conjugated goat anti-rabbit antibody (50 μl) was added to each slide and incubated at room temperature for 10 min, washed with PBS for 3x5 min, dried and observed under a fluorescence microscope.

Specific fluorescence intensities were classified as follows: -, no fluorescence; ±, weak fluorescence; +, clear fluorescence; ++, low fluorescence; and +++/++++, strong fluorescence.

Statistical analysis. Data were analyzed using SPSS software, version 11.5 (SPSS Inc., Chicago, IL, USA) and expressed as means ± standard deviation. Enumeration data were analyzed using exact probability of 4-fold table and measurement data with completely random analysis of variance. A 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, the rats exhibited rapid breathing and the symptoms were aggravated with time, followed by discoloration of the skin and mucosae (cyanosis), unconsciousness and occasionally death (the death rate was 20% in the model group).

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 hemorrhage and necrosis and bloody ascites were observed in the surviving rats of the model group. In addition to the appearance of yellow saponaceous spots on the greater omentum and common bile duct, pulmonary hyperemia, edema and hemorrhage, gastric edema and paralytic expansion, hepatic swelling and renal augmentation were observed. However, in the rats in the sham surgery group, only a mild edema of the gastrointestinal mucosa and exudation in the abdominal cavity were observed.

Table I. Intercellular adhesion molecule-3 expression in each group as detected by flow cytometry (mean ± standard deviation).

Groups	n	Expression rate (%)
Sham surgery	10	26.12±3.32
Control	8	12.57±5.37[a]
Emodin	8	22.40±2.25[b]
Dexamethasone	8	16.64±5.36[b,c]

[a]P<0.05 vs. sham surgery group; [b]P<0.05 vs. control group; [c]P<0.05, vs. emodin group.

Pathological changes in pancreatic tissues observed under a light microscope. The sham surgery group exhibited a distinct pancreatic lobular structure. The model group presented with necrosis in the pancreatic glandular parenchyma, bleeding and fatty degeneration, erythrocyte stasis, angiectasis and PMN infiltration of the interstitial space and parenchyma.

MΦ ICAM-3 expression. The ICAM-3 expression in each group as detected by flow cytometry (mean ± standard deviation) is presented in Table I and Fig. 2. The expression of ICAM-3 was significantly decreased in the control group, whereas the expression of ICAM-3 was significantly increased in the emodin and dexamethasone groups. Of note, the expression of ICAM-3 was significantly increased in the emodin group.

pMΦ phagocytosis rate and phagocytic index. The positively stained pMΦs were considered to be those that phagocytized PMNs and were quantified by microscopy (magnification, 40). Three regions were randomly selected, 100 cells were quantified and the mean was calculated. The phagocytosis rate and phagocytic index were calculated to evaluate phagocytotic ability.

The phagocytosis rate and phagocytic index were significantly lower in the control group, alhtough they were increased in the emodin and dexamethasone groups. Of note, the phago-
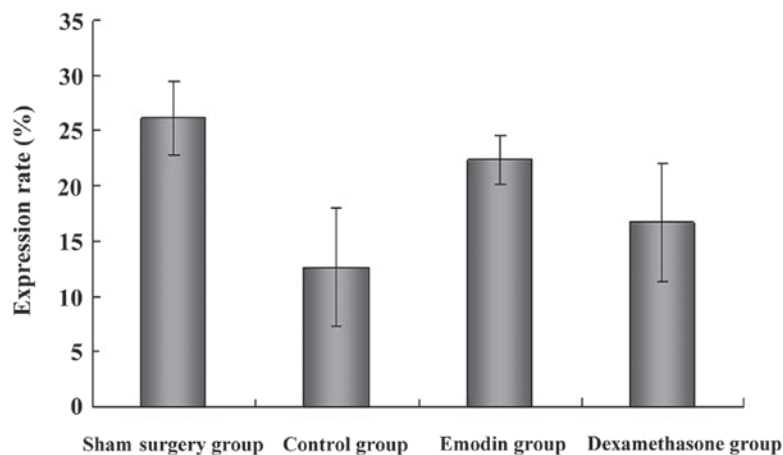

Figure 2. Intercellular adhesion molecule-3 (ICAM-3) expression in each group as detected by flow cytometry. ICAM-3 expression was significantly decreased in the control group and increased in the emodin and dexamethasone groups. Of note, ICAM-3 expression was significantly increased in the emodin group.
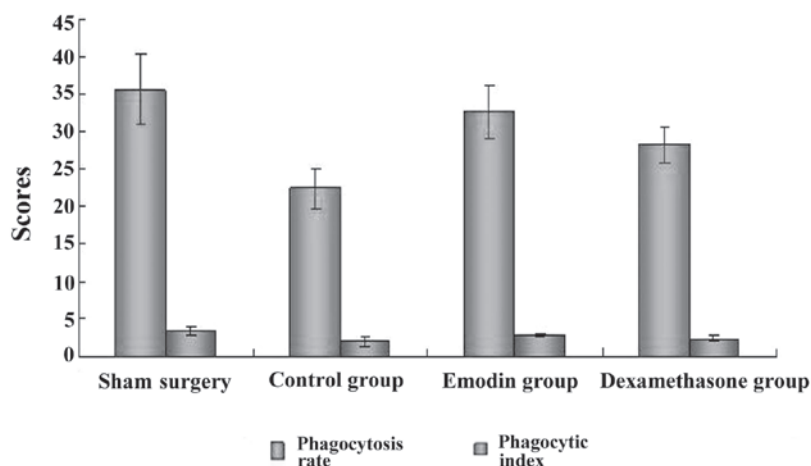
Figure 3. Peritoneal macrophage phagocytosis rate and phagocytic index in each group. The phagocytosis rate and phagocytic index were significantly lower in the control group, whereas they were increased in the emodin and dexamethasone groups. Of note, the phagocytosis rate and phagocytic index were significantly increased in the emodin group.

Table II. Peritoneal macrophage phagocytosis rate and phagocytic index in each group (mean ± standard deviation).

Groups	n	Phagocytosis rate (%)	Phagocytic index
Sham surgery	10	35.6±4.8	3.4±0.6
Control	8	22.4±2.7[a]	2.0±0.5[a]
Emodin	8	32.6±3.5[b]	2.8±0.2[b]
Dexamethasone	8	28.2±2.4[c]	2.4±0.3[c]

[a]P<0.05 vs. sham surgery group; [b]P<0.05 vs. control group; [c]P<0.05 vs. emodin group.

cytosis rate and phagocytic index were significantly increased in the emodin group (Table II and Fig. 3).

MΦ ICAM-3 protein expression. Immunofluorescence staining demonstrated that ICAM-3 was mainly expressed on the cell membrane. There were statistically significant differences between the control and sham surgery groups, between the emodin and control groups and between the dexamethasone and control groups (P<0.05; Table III).

Discussion

PMNs are crucial for the control of infection. However, the pathogenesis of inflammation is associated with PMNs and their toxic content release. Following necrosis or disaggregation, PMNs may release a large amount of toxins, which induce tissue damage, a cascade reaction and SIRS (15). Under normal conditions, the half-life of PMNs is 6-10 h; however, after PMNs enter inflammatory sites, their half-life is significantly prolonged (16,17). Spontaneous apoptosis occurs in senescent PMNs, even in the absence of cytokines or under pro-inflammatory conditions. Apoptotic PMNs may be completely phagocytized, thereby preventing toxin release that may induce tissue injury. Moreover, MΦ phagocytosis of apoptotic PMNs does not stimulate the release of inflammatory mediators (18,19). Therefore, apoptosis is considered to be an effective physiological adjustment for the clearance of PMNs (20). Following acute inflammation, PMNs migrate from the circulation to inflammatory sites, significantly increasing the numbers of PMNs in the tissues. Moreover, inflammation reduces PMN apoptosis, resulting in PMN activation and SIRS.

MΦs exhibit a strong phagocytotic capacity. Recent evidence indicated that after MΦs phagocytize apoptotic PMNs, transforming growth factor (TGF)-β1 is released, which inhibits the production of inflammatory factors

Table III. Expression of intercellular adhesion molecule-3 (ICAM-3) in the sham surgery, emodin and dexamethasone groups compared to the control group.

Groups	n	ICAM-3 expression				Positive rate (%)	P-value
		-	+	++	+++		
Sham surgery	10	2	3	4	1	80	0.016
Emodin	8	1	3	4	0	87.5	0.008
Dexamethasone	8	2	2	4	0	75	0.03
Control	8	5	3	0	0	37.5	-

-, no fluorescence; +, clear fluorescence; ++, low fluorescence; +++, strong fluorescence.

interleukin (IL)-1β, tumour necrosis factor α (TNF-α) and granulocyte-macrophage colony-stimulating factor (19,21-23). Following apoptosis, the phagocytes recognize and phagocytize apoptotic cells and bodies with an intact membrane structure. The process of MΦ phagocytosis of apoptotic cells is 'quiet' and is not accompanied by cytolysosome, mitochondrion and membrane breakage, preventing cell content release, inflammatory reaction and tissue injury. Therefore, the phagocytotic capacity of MΦs is crucial for the containment of inflammation. If the mechanism by which MΦs recognize and phagocytize apoptotic cells is damaged, apoptotic cells cannot be phagocytized, which leads to toxic content release and subsequent tissue and cell injury (18).

Biochemical and membrane changes in apoptotic cells may be the basis by which MΦs recognize and phagocytize apoptotic cells. MΦs express various receptors that identify apoptotic cells and apoptotic cells possess corresponding markers to exhibit their 'edibility'. However, the interaction between selection and phagocytosis has not been fully elucidated.

In the present study, the phagocytotic capacity of pMΦs was shown to be reduced following SAP/SIRS, which may contribute to delayed PMN apoptosis and necrosis, as well as toxic content and chemotactic factor release, which inhibit 'edibility' signal expression. Moreover, the membrane structure and internal environment are altered by toxic content and chemotactic factor release, which may also attenuate the phagocytotic capacity of pMΦs.

ICAM-3 is a member of the immunoglobulin superfamily and is involved in cell adhesion and signal transduction. It is mainly expressed by leukocytes and highly expressed by lymphocytes, monocytes and neutrophilic granulocytes (24). ICAM-3 is also expressed on MΦs (24).

In the present study, pMΦ ICAM-3 protein expression was shown to be significantly decreased following SAP/SIRS compared to that in the control group. The likely mechanism is that SIRS induces organ and cell injury; thus, MΦs synthesize and release a large amount of TNF-α and other inflammatory mediators that trigger inflammatory reactions. TGF-β and IL-4 downregulate MΦ ICAM-3 expression, thereby inhibiting its interaction with CD14, which affects phosphatidylserine externalization and reduces the phagocytotic capacity of MΦs. Previous studies demonstrated that the delayed apoptosis of human neutrophils may be attributed to the disturbed identification of MΦs under pathological conditions (4,12).

Da Huang (rhubarb), a Chinese herb, was demonstrated to be clinically effective for the treatment of acute pancreatitis (25-28). Emodin is the main active component of Da Huang. Previous studies demonstrated that emodin affects bacteriostasis, catharsis, relieves Oddi sphincter spasm, inhibits abnormal metabolism of vasoactive substances (e.g., eicosenoic acid), improves the microcirculation and antagonizes coagulation and thrombus formation (25,29,30). The present study demonstrated that emodin significantly increases MΦ ICAM-3 protein expression in rats with SAP/SIRS (P<0.05), thereby promoting the interaction between ICAM-3 and CD14, enhancing identification and phagocytotic capacity of pMΦs and relieving inflammatory reactions.

Stephenson *et al* (31) first reported the application of glucocorticoids in the treatment of acute pancreatitis. However, the mechanism has not been fully elucidated. The effects of gluco-corticoids on inflammation via receptor mediation may trigger anti-inflammatory processes. Glucocorticoids inhibit inflammatory exudation, leukocytic infiltration and inflammatory mediator production and release, improve the microcirculation, alleviate endotoxemia and induce apoptosis of pancreatic acinar cells, thereby reducing the degree of pancreatic necrosis in SAP (32,33). A previous study demonstrated that pancreatic cell apoptosis occurs during acute pancreatitis (34). Animal experiments also suggested that dexamethasone induces pancreatic cell apoptosis, stabilizes the internal environment and attenuates inflammation in pancreatic tissues (33,35). In the present study, dexamethasone enhanced the phagocytotic capacity of pMΦs, maintained the internal environment and increased MΦ ICAM-3 expression.

In conclusion, the capacity of pMΦs to phagocytize apoptotic PMNs is significantly reduced following SAP/SIRS. This effect may be associated with decreased pMΦ ICAM-3 expression. Emodin and dexamethasone were shown to enhance the phagocytotic capacity of pMΦs, possibly by increasing pMΦ ICAM-3 expression. Of note, emodin exerted more potent effects compared to dexamethasone.

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