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 Ca²⁺ 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
(SAP/SIRS) groups (n=30). pMΦs 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 (FACSAria) 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; and dianisidine (FITC)-conjugated goat anti-rabbit antibody at room temperature for 30 min. Non-phagocytized PMNs 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.

**Isolation, purification, culture and administration of pMΦs.** Trypan blue staining revealed that the PMN survival rate and purity were >95%. Isolated PMNs were seeded at 5x10^6 cells/ml in u-well culture plates and incubated at 37°C in a humidified atmosphere with 5% CO₂ 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% CO₂ 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% H₂O₂ 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:

\[
\text{Phagocytosis rate} = \frac{\text{Number of pMΦs phagocytizing PMN}}{100 \text{ pMΦ}} \times 100\% \text{ (mean of three regions)}
\]

\[
\text{Phagocytic index} = \frac{\text{Number of phagocytized MΦs}}{100 \text{ pMΦ}} \text{ (mean of three regions)}
\]

**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% CO₂ 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% CO₂ 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 mean ± 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>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Expression rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham surgery</td>
<td>10</td>
<td>26.12±3.32</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>12.57±5.37</td>
</tr>
<tr>
<td>Emodin</td>
<td>8</td>
<td>22.40±2.25</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>8</td>
<td>16.64±5.36</td>
</tr>
</tbody>
</table>

*P<0.05 vs. sham surgery group; *P<0.05 vs. control group; *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.

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 phagocytic ability.

The phagocytosis rate and phagocytic index were significantly lower in the control group, although they were increased in the emodin and dexamethasone groups. Of note, the phago-
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 can 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 inflammatory mediators (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 phagocytic capacity. Recent evidence indicated that after MΦs phagocytize apoptotic PMNs, transforming growth factor (TGF)-β1 is released, which inhibits the production of inflammatory factors

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Phagocytosis rate (%)</th>
<th>Phagocytic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham surgery</td>
<td>10</td>
<td>35.6±4.8</td>
<td>3.4±0.6</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>22.4±2.7*</td>
<td>2.0±0.5*</td>
</tr>
<tr>
<td>Emodin</td>
<td>8</td>
<td>32.6±3.5*</td>
<td>2.8±0.2*</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>8</td>
<td>28.2±2.4*</td>
<td>2.4±0.3*</td>
</tr>
</tbody>
</table>

*P<0.05 vs. sham surgery group; *P<0.05 vs. control group; *P<0.05 vs. emodin group.

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>-</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>Positive rate (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham surgery</td>
<td>10</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>80</td>
<td>0.016</td>
</tr>
<tr>
<td>Emodin</td>
<td>8</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>87.5</td>
<td>0.008</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>75</td>
<td>0.03</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>37.5</td>
<td>-</td>
</tr>
</tbody>
</table>

-, 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 cytokylosome, mitochondrion and membrane breakage, preventing cell content release, inflammatory reaction and tissue injury. Therefore, the phagocytic 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 phagocytic 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 phagocytic 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 phagocytic 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., eicosanoic 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 phagocytic 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 glucocorticoids 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 endotoxinemia 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 phagocytic 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 phagocytic capacity of pMΦs, possibly by increasing pMΦ ICAM-3 expression. Of note, emodin exerted more potent effects compared to dexamethasone.

References


