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## **MMP-9, MMP-2 Profile in Different Mice Tissues and LPS Induced MMP-9 and MMP-2 Expression in RAW 264.7 Cells, Down Regulated by Emodin Treatment**

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### **Abstract**

Emodin, an anthraquinones component of Rheum palmatum, has been used for anti-inflammatory purposes. However, its underlying molecular effect(s) on target cells remain to be well clarified. our current study was aimed at investigating the regulatory effect of Emodin on lipopolysaccharide induced inflammatory responses in RAW 264.7 macrophages by gelatin zymography . It was found that treatment of LPS(5µg/ml) for 18 h elevates the expression of MMP-9 and MMP-2 proteins in RAW 264.7 cells. It was also found that co-treatment of RAW 264.7 with drug Emodin(6µg/ml) and LPS(5µg/ml) for the period of 18 h reduces the expression level of MMP-9 and MMP-2 protein. Our data suggest that Emodin plays roles in regulating the digestion of Extracellular matrix by down-regulating the expression of MMP-9 and MMP-2 proteins.

**Key words:** MMP-9, MMP-2, RAW 264.7 cell line, lipopolysaccharide,

### **Intrduction**

Emodin(1,3,8 trihydroxy-6-methyanthraquin- one ) is a major component in the roots of rhubarb (Rheum palmatum L.) (1-3). Emodin possesses anticancer, anti bacterial, diuretic and vasorelaxant effect (4-6). Strikingly, it has been reported that Emodin has the potential to inhibit several angiogenesis processes(7,8).

Matrix metalloproteinases(MMPs), are a family of neutral endopeptidases that are involved in angiogenesis, embryogenesis, normal tissue remodeling and repair. MMPs secrete in a latent form (pro MMPs).The latent MMPs require an activation step before they are able to cleave extracellular matrix (ECM) component. Dysregulated expression of MMPs leads to the destruction of connective tissues and various pathological inflammatory conditions (9).For example MMPs are involved in tumor invasion and metastasis(10,11).MMPs are crucial in inflammatory cells , including macrophages , lymphocytes , neutrophils and eosinophils(12).Macrophages are prominent at chronic inflammatory diseases such as areas of arthritis , arteriosclerosis and periodontal diseases in which the degradation of the connective tissue is believed to contribute substantially to the pathology of the disease. Stimulation of macrophages with LPS, induces the expression of number of MMPs, including two prominent macrophage MMPs: MMP-2 and MMP-9. MMP-2 is often over-expressed in highly metastatic tumors, whereas MMP-9 can be stimulated by an inflammatory cytokine (13), a growth factor (14,15), or an oncogene (14,15) through activation of different intracellular-signaling pathways. It was reported that MMP-2 and MMP-9 are capable of degrading most extracellular matrix (ECM) components that form the basal membrane (16). Numerous reports have been demonstrated that inhibition of MMP expressions or enzyme activities may

be considered to be early targets for preventing cancer metastasis (17,18). Therefore, agents possessing the ability to suppress the expression of MMP-2 or -9 are worthy for the development of therapies for inflammation, cancer invasion and metastasis.

In this study, we investigated the effects of LPS affecting RAW 264.7 cell metabolism on the regulation of MMP-2 and MMP-9 expression, we also analyzed whether Emodin could effectively suppress the effect of LPS by controlling the expression of MMP-2 and MMP-9 expression in RAW 264.7 cells. Taken together, our results indicate that Emodin attenuates the expression of MMP-2 and MMP-9 that are up-regulated in LPS induces inflammations, inflammatory diseases and cancer cells and thus could become a potential anti inflammatory and anti cancer drug candidate.

## **Methods:**

### **Chemicals.**

LPS (endotoxin from *Escherichia coli*), drug Emodin

### **Tissue sample preparation.**

Healthy mouse was sacrificed and tissues were isolated as per ethical guidance. The tissues (liver, kidneys, brain and lungs) were immediately snap frozen in liquid nitrogen and stored in -80°C till use. Weighed amount of tissues were homogenized in homogenizing buffer (PBS+1% triton) using Teflon homogenizer at 8000rpm for 2 min. the homogenate was cleared by centrifuging at 10,000 rpm for 10 min. and the supernatant collected was used for zymography.

### **Cell cultures.**

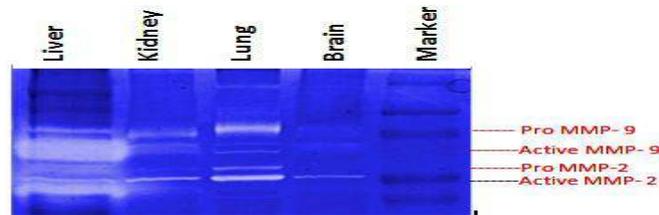
RAW 264.7 cell lines were grown in Dulbecco's modified Eagle medium supplemented with 10% (v/v) FBS, penicillin 100 (U/ml), streptomycin (100 µg/ml). For stimulation assays, cells were trypsinized with a solution of 1 mM EDTA/0.25% (w/v) trypsin, seeded at a density of  $1 \times 10^5$  cells/ml in 24-well plate, and incubated for 12 h at 37°C in 5% CO<sub>2</sub>. Monolayers were then washed three times with phosphate buffer saline (PBS), and fresh media was added with appropriate concentration of LPS and/or drug Emodin. Supernatant was collected after 18 h of stimulation and stored at -20°C until assayed.

### **Determination of MMP-9 by Zymography.**

MMP in the medium released from RAW264.7 cells was assayed using gelatin zymography (10% zymogram gelatin gels) according to the methods reported by S P Hawkes et al. (19) with some modification. Briefly, the culture medium was electrophoresed (120 V for 90 min) in a 10% SDS-PAGE gel containing 0.1% gelatin. The gel was then washed at room temperature in a solution containing 2.5% (v/v) Triton X-100 and subsequently transferred to a reaction buffer for enzymatic reaction containing 1% NaN<sub>3</sub>, 10mM CaCl<sub>2</sub> and 40 mM Tris-HCl, pH 8.0, at 37 °C with shaking overnight (for 12–15 h). Finally, the MMP gel was stained for 30 min with 0.25% (w/v) Coomassie blue in 10% acetic acid (v/v) and 20% methanol (v/v) and destained in 10% acetic acid (v/v) and 20% methanol (v/v).

## Results

**A) Tissue homogenate.** After the centrifugation of tissue homogenates, the supernatants were collected and the aliquots were mixed with non-reducing sample buffer in 1:1 ratio. Prior to loading the samples were heated at 55 °C for 4 min. After the gel run was completed, the gel was developed overnight in 37°C incubator followed by fixing, staining and then destaining. The following results were obtained after following the method of gelatin zymography.



**Figure 1.** The 10% gelatin zymograph showing the activity of matrix metalloproteinase (MMP/MMP-9 and MMP-2) in different tissue samples.

### Lanes

1. liver sample
2. kidney sample
3. lung sample
4. brain sample.

The above zymographic gel is to provide to validate the specificity of the assay to detect latent and active forms of the MMPs. Tissue samples contained several protease activities at molecular weights of 92, 84, 72, and 64 kD. The upper gelatinolytic bands represent the 92 kD (latent or pro form) and 88 kD (active form) MMP-9 species, whereas the lower gelatinolytic bands represent the 72 kD (latent or pro form) and 64 kD (active form) MMP-2 species. The intensity of the Mr72,000 and 64,000 bands was much higher in liver sample than other samples. In addition, the levels of the proMMP-9 and active MMP-2 were also significantly higher in lung sample than other samples.

### B) Cell supernatant

**1. MMP expression profile in stimulated RAW 264.7 cell supernatant (stimulated by LPS).** The gelatinolytic activity were assessed in the two samples and gelatin zymography was performed in RAW cell supernatant. Fig c. reports the results of a representative zymographic analysis of two samples. In this experiment, the samples were heated at 50 °C for 5 mins, 20 µl of samples were loaded in each well and the gel was developed overnight at room temperature.



**Figure 2. Gelatin zymography.** 10% gelatin gel showing the comparison of MMP profile in RAW cell line.

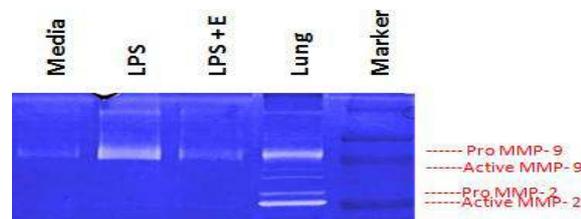
### Lanes

1. C, zymogram of supernatant of unstimulated RAW cell suspension

2. Lps, zymograph of RAW protein sample of cultured media stimulated with LPS (1 $\mu$ g/ml)

**LPS induced MMP expression.** Fig 2. showed the MMP expression in RAW 267.4 cells, and LPS enhanced its expression. We examined the MMP activity, released from LPS-treated cells using gelatin zymography assays. After stimulating RAW cells with LPS, biologically active MMP in cell culture supernatant increased. The result showed that LPS stimulated RAW 264.7 cells release biologically active MMP protein into medium.

2. **Lower conc. of LPS for shorter period of time(12 h) induced expression of MMP-9 in RAW 264.7 cell line while stimulation reduced by co-treatment with drug Emodin.** To determine whether LPS induces expression of MMP-9 in RAW macrophages, cells were cultured in the presence of LPS and LPS+Emodin for the period of 12 hrs and the supernatants were subjected to gelatin zymography for MMP detection. Fig e. showed the MMP-9 expression in RAW 264.7 cell supernatant. We examined that LPS induced the expression of MMP-9 and drug Emodin reduced the stimulation of RAW cells in presence of LPS.



**Fig 3.** Detection of MMP-9 activity in four samples of using 10% zymography.

### Lanes

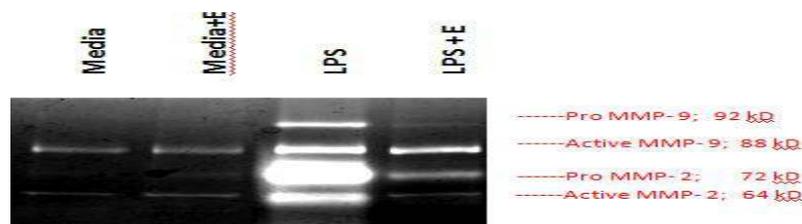
1. C, zymogram of protein samples of culture media of RAW cells.

2. Lps, zymogram of protein sample of culture media of RAW cells stimulated with LPS(1 $\mu$ g/ml)

3. D, zymogram of protein sample of culture media of RAW cells treated with Lps and drug Emodin

4. Lg, zymogram of lung tissue sample of healthy mice

3. **Higher conc. of LPS (5 $\mu$ g/ml) for longer period of time (18 h) triggers the expression of both MMP-9 and MMP-2 in RAW cell supernatant while the co-treatment of LPS(5 $\mu$ g/ml) and drug Emodin(6 $\mu$ g/ml)inflammatory response reduces the expression of both MMP-9 and MMP-2 in RAW 264.7 cell lines.**



**Fig 4.** Emodin suppresses LPS induced MMP activities. The conditioned media were collected MMP activities determined by gelatin zymography. MMP activities were quantified by densitometric analysis.

### Lanes

1. **C1**, zymogram of protein samples of culture media of RAW cells.
2. **C2**, zymogram of protein samples of culture media of RAW cells treated with drug Emodin.
3. **Lps**, zymogram of protein sample of culture media of RAW cells stimulated with LPS (5 $\mu$ g/ml).
4. **D**, zymogram of protein sample of culture media of RAW cells treated with LPS and drug Emodin.

The gelatinolytic bands present in LPS treated lane showed much clear bands compared to the control. This indicates that LPS (5 $\mu$ g/ml) treatment for a period of 18 hrs increased MMP-2 and MMP-9 expression in RAW cell line compared to that in the untreated cells. Treatment of Emodin at a concentration of 6  $\mu$ M decreased the LPS induced protein level of MMP-2 and MMP-9.

### Discussion

In the present study, we demonstrated activities of Emodin in *in vitro* experimental systems, by using LPS-stimulated RAW264.7 macrophages. The pathology is initiated by complex processes triggered by microbial pathogens such as LPS which is a prototypical endotoxin. In this study, it is found that LPS stimulated MMP-9 up-regulation predominates at low LPS conc.(1 $\mu$ g/ml, figure 3), while the expression of both MMP-9 and MMP-2 is up-regulated at high LPS conc.(5 $\mu$ g/ml, fig 4) and the treatment of Emodin effectively reduces the expression of MMP-9 and MMP-2 in LPS induced inflammatory cell, while it is more effective in reducing the expression of MMP-2 in LPS treated cells.

Of considerable interest in this study was marked decrease by Emodin the secretion of MMP-2 and MMP-9 from LPS-stimulated RAW cells as determined by zymography. These findings suggest Emodin may have anti-inflammatory and anti cancerous effects through the inhibition of MMP-2 and MMP-9 expression, which has been linked to the metastasis of cancers by the digestion of extra cellular matrix, as the cancer cells over express MMP-9 and MMP-2 proteins. Tumor-secreted MMPs destroy extracellular matrix components in tissue surrounding a tumor, enter and survive in the circulation, lymphatic or peritoneal spaces and can arrest in a distant target organ. Herein, it can be revealed that Emodin could significantly inhibit the invasive and migration ability of cancer cells. In the present study, we show that Emodin could inhibit protein levels of tumor metastasis related proteins such as MMP-2 and MMP-9. Thus, the possible usefulness of specifically selected MMP inhibitor, would be worthy of investigation as therapeutic agents that prevent the metastasis of cancers. Emodin can be targeted as an effective anti metastatic agent for tumor progression. Therefore, the inhibitory effect of Emodin could make it a perfect fit for anti cancerous and anti inflammatory therapy.

Figure 1. demonstrates the differential expression of MMP-9 and MMP-2 in different mice tissues, the expression is highest in liver sample than lung, kidney and least in brain tissue that may be prerequisites for understanding of their role in repair, remodeling and disease pathogenesis.

This study is expected to provide clues that the drug Emodin may have therapeutic potential for the treatment of cancers and other inflammatory diseases.

## Conclusion

Formation and expression of MMPs play a significant role in inflammation and digestion of extracellular matrix. First we examined the expression of MMPs in untreated samples. We also examined the expression of MMP-9 and MMP-2 in LPS (5µg/ml) treated cells and its subsequent inhibition in Emodin (6 µM) treated cells. We confirmed our results at the protein level using gelatin zymography in three different conditions such as untreated, LPS treated and LPS treated+Emodin. Increased gelatinolytic activity of MMP-2 and MMP-9 in LPS treated culture supernatant shows their higher expression and that play an important role in the digestion of extracellular matrix components. Decreased gelatinolytic activity in Emodin treated cells shows the involvement of Emodin in the down regulation of MMP2 and MMP9 proteins. MMPs are crucial in the progression of cancers. Drug Emodin showed the down regulation of MMPs expression and thereby inhibits cancer cell migration. Further characterization of Emodin mediated down regulation of MMP-2 and MMP-9 is an important subject for future research.

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