EXPERIMENTAL STUDY

The Role of FAK in the Secretion of MMP9 after CD147 Stimulation in Macrophages

Chen Yu,1,2 MD, Yang Lixia,2 MD, Guo Ruiwei,2 MD, Shi Yankun,2 MD and Ye Jinshan,2 MD

Summary
To investigate whether focal adhesion kinase (FAK) can participate in the secretion of matrix metalloproteinase 9 (MMP9) after CD147 stimulation in THP-1 induced macrophages; thus, to explore the potential treatment perspectives for acute coronary syndrome (ACS).

Phorbol-12-myristate-13-acetate (PMA) was used to induce THP-1 cells to differentiate into macrophages. To confirm the peak mRNA and protein expression of FAK and MMP9 after the stimulation of CD147, the macrophages were divided into 5 groups (0, 3, 6, 9, and 12 hours), with 0 hours group as control group. To investigate the role of FAK in the secretion of MMP9, with stimulation of CD147 for 9 hours, FAK inhibitor 14 was used to inhibit FAK Y397 phosphorylation. The mRNA and protein expressions were quantified by qRT-PCR and western blotting, respectively.

(1) Relative mRNA expression of FAK and MMP9 were both significantly up-regulated (all \( P < 0.05 \)) after stimulation of CD147, FAK peaked at 9 hours (3.908 ± 0.106 versus 1, \( P < 0.05 \)), whereas MMP9 peaked at 6 hours (2.522 ± 0.062 versus 1, \( P < 0.05 \)). (2) Relative protein expression of FAK, pFAK, and MMP9 were all significantly increased after CD147 stimulation (all \( P < 0.05 \)), FAK (1.930 ± 0.024 versus 1, \( P < 0.05 \)) and pFAK (1.737 ± 0.021 versus 1, \( P < 0.05 \)) peaked at 9 hours, whereas MMP9 peaked at 6 hours (1.527 ± 0.033 versus 1, \( P < 0.05 \)). (3) CD147 up-regulates FAK, pFAK, and MMP9 mRNA and protein expressions in a dose-dependent manner. (4) FAK inhibitor 14 significantly reduced the relative protein expression level of pFAK (0.077 ± 0.012 versus 1, \( P < 0.05 \)) and MMP9 (0.133 ± 0.012) at 9 hours after CD147 stimulation.

The results demonstrated that FAK Y397 phosphorylation was involved in the secretion of MMP9 after CD147 stimulation in macrophages and may play a role in the regulation of ACS.

Key words: Antigens, Focal adhesion protein-tyrosine kinases, Acute coronary syndrome

Acute coronary syndrome (ACS) is one of the leading causes of mortality, even if there has been a great improvement in the treatment. The formation of thrombus as the progression of vulnerable plaque is an important cause of acute coronary syndromes. The key features of vulnerable plaques include large necrotic core, thin fibrous cap, and abundant foam cells. Great advances have been made in the signaling pathways involved in the formation of unstable plaques for developing therapeutic methods. However, despite the considerable efforts, the mechanisms underlying the vulnerable plaques remain incompletely understood.

The imbalance, which leads to plaque rupture, is between the extracellular matrix (ECM) deposition and matrix destruction. CD147, first identified in tumor cells and also known as extracellular matrix metalloproteinase inducer (EMMPRIN), can induce the production of various metalloproteinases (MMPs). MMPs are a family of zinc-dependent proteinases capable of degrading various structural components of ECM, thus leading to ECM destruction and plaque rupture. These findings motivate further investigation of the mechanism of MMPs secretion in ACS. MMP9, secreted by macrophages, is used as an important marker for detecting vulnerable plaques.

Focal adhesion kinase (FAK) is a protein tyrosine kinase (PTK), activated by cell attachment to the extracellular matrix and plays a role in regulating cell attachment and motility. Wu et al. found that MMP2 expression was elevated after CD147 stimulation in hepatocellular carcinoma, together with FAK and pFAK protein expression. Therefore, FAK might be involved in secretion of MMP9 in macrophages.

To shed light on potential effects of FAK on MMP9 secretion in macrophages, FAK and MMP9 mRNA and protein expression were analyzed after CD147 stimulation. In addition, with FAK inhibitor 14 approach, an essential...
role of FAK was demonstrated in the regulation of MMP9 secretion.

Methods

Cell culture and differentiation: THP-1 cells were purchased from Kunming Institute of Zoology. To differentiate THP-1 cells into macrophages, THP-1 cells were plated in RPMI 1640 media (Hyclone, China) with 10% fetal bovine serum (Hyclone, China) in CO2 at 37°C. THP-1 cells (1×10⁶/mL) were induced to differentiate into macrophages using 5 ng/mL phorbol-12-myristate 13-acetate (PMA) (Solarbio, China) for 48 hours. Differentiation of PMA treated cells was enhanced after the initial 48 hours stimulus by removing the PMA-containing media and then incubating the cells in fresh RPMI 1640 for 24 hours. To explore the peak of MMP9 and FAK after CD147 (Abcam, USA) stimulation, all the FAK and MMP9 mRNA expression were up-regulated, FAK peaked at 9 hours and MMP9 peaked at 6 hours (Figure 2).

Western blotting analysis: Cells were lysed in Cell lysis buffer for western blot (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100) (Beyotime, China) containing complete protease and phosphatase inhibitor cocktail. Protein concentration was determined by the Enhanced BCA Protein Assay Kit (Beyotime, China), and gels were loaded with equal amounts of protein per lane. Electrophoretic separation was carried out on 7% polyacrylamide gels (Beyotime, China), and subsequently transferred to PVDF membrane (Beyotime, China). Membranes were blocked in 5% non-fat dry milk powder in TBST buffer for 1 hour. Then the membranes were incubated overnight at 4°C with primary antibodies: rabbit anti-FAK (Y695) monoclonal antibody (1:1000) (Abcam, USA), rabbit anti-FAK (Y397) monoclonal antibody (1:1000) (Abcam, USA), rabbit anti-MMP9 monoclonal antibody (1:1000) (Abcam, USA), followed by incubation in secondary antibodies: HRP-Goat Anti-Rabbit IgG (H + L) (1:2000) (Proteintech, China). GAPDH (1:2500) (Abcam, USA) served as the loading control. ECL detection system (Beyotime, China) was used to detect protein bands.

Statistical analysis: Data from three independent experiments were presented as mean ± SD. Two-sided Student’s t-test was used to analyze the different protein expression of FAK, pFAK, and MMP9 after FAK inhibition. All statistical analyses were conducted using the SPSS 20.0. P < 0.05 was considered statistically significant.

Results

The FAK inhibitor 14 inhibits viability of macrophages in a time-dependent manner: To determine whether the FAK inhibitor 14 inhibits viability of macrophages in a time-dependent manner, CCK8 assay was performed with different time: 0, 3, 6, 9, and 12 hours of FAK inhibitor 14 at 10 μmol/L. The viability of macrophages started to decrease at 6 hours (Figure 1).

CD147 up-regulates FAK and MMP9 mRNA expression: To test the effect of CD147 on FAK and MMP9 mRNA expression, the macrophages were treated with CD147 at 1 ng/mL for 0, 3, 6, 9, and 12 hours, and then qRT-PCR was performed. The result shows that after CD147 stimulation, all the FAK and MMP9 mRNA expression were elevated, FAK peaked at 9 hours and MMP9 peaked at 6 hours (Figure 2).

CD147 up-regulates FAK and MMP9 protein expression: To explore the effect of CD147 on FAK and MMP9 protein expression in a time-dependent manner, the macrophages were treated with CD147 at 1 ng/mL, for 0, 3, 6, 9, and 12 hours, and then western blot was used. The result shows that all FAK and MMP9 protein expression were up-regulated, peaked at 9 hours and peak at 6 hours, respectively (Figure 3).

CD147 up-regulates FAK and MMP9 mRNA expression in a dose-dependent manner: To test whether
CD147 has an effect on FAK and MMP9 mRNA expression in a dose-dependent manner. The macrophages were treated with CD147 at 0, 0.1, 0.5, 1, 5, 10 ng/mL for 9 hours, with 0 ng/mL as control groups, and then qRT-PCR was performed. The result shows that both FAK and MMP9 mRNA expression peaked at 1 ng/mL CD147 stimulation (Figure 4).

**CD147 up-regulates FAK and MMP9 protein expression in a dose-dependent manner:** To explore whether CD147 has an effect on FAK and MMP9 protein expression in a dose-dependent manner, the macrophages were treated with CD147 at 0, 0.1, 0.5, 1, 5, 10 ng/mL for 9 hours, and then western blot was performed. The result shows that both FAK and MMP9 protein expression peaked at 1 ng/mL CD147 stimulation for 9 hours (Figure 5).

The **FAK inhibitor 14 blocks FAK Y397 phosphorylation and down-regulates MMP9 protein expression:** To confirm whether FAK signaling participates in the secretion of MMP9 after CD147 stimulation, the macrophages were treated with CD147 at 1 ng/mL and FAK inhibitor 14 at 10 μM for 9 hours. The result shows that FAK inhibitor 14 significantly decreased FAK Y397 phosphorylation, together with MMP9 (Figure 6).

**Discussion**

Previous reports have shown that macrophages participate in the development of atherosclerosis. CD147 up-regulates MMPs through several signaling pathways, including JAK/STAT, Ras-MEK1-MAPK, and PI3k/Akt signaling pathway, thus leading to the instability of the plaque. In the present study, FAK was revealed to play an essential role in MMP9 secretion after CD147 stimulation in macrophages. In the beginning, THP-1 cells were differentiated into macrophages using PMA, followed by the stimulation from CD147. After that, both FAK and MMP9 had their mRNA and protein expressions elevated, which were assessed by qRT-PCR and western blot. Then, CD147 up-regulated FAK, pFAK, and MMP9 in a dose-dependent manner. At last, FAK inhibitor 14 was used to block FAK phosphorylation, and it was revealed that down-regulation of FAK phosphorylation could attenuate MMP9 secretion after FAK inhibitor 14 was used to block FAK phosphorylation.

Postmortem reports of coronary artery findings in ACS include plenty of macrophages. The more macrophages invaded into the plaque, the more vulnerable plaques became. This is due to excess MMPs secretion of macrophages responding to CD147, thus resulting in de-
grading ECM, weakening fiber cap and the plaque rupture. Therefore, excess MMPs secretion of macrophages is the key factor of acute coronary syndrome. And how to reduce MMPs secretion in the macrophages or decrease activity of MMPs has been paid great attention to. However, many studies show that statins, ACEI or ARB, aspirin can decrease activity of MMPs in different ways, the famous PROVE indicated that the recurrence rate of
ACS was 22% under the combination of statins, ACEI or ARB and aspirin. Present study for the first time demonstrated that blocking FAK phosphorylation can lead to the reduction of MMP9 secretion after CD147 stimulation in macrophages and may shed new lights on the treatment of ACS.

FAK plays an important role in the pathogenesis of many human diseases. However, it is yet not clear whether FAK is involved in the regulation of MMP9 secretion in response to CD147 stimulation in macrophages. Present study shows that CD147 stimulation can up-regulate MMP9 secretion in macrophages, together with FAK and pFAK. By inhibition of phosphorylation of tyrosine kinase 397 in FAK, the FAK inhibitor 14 reduces MMP9 secretion. This indicates that FAK is essential for regulation of MMP9 secretion in macrophages.

However, some research limitations still exist in the present work. On one hand, the downstream targets of FAK and exact molecular mechanism are still unclear. On the other hand, it is yet not clear whether blocking FAK can reduce MMP9 secretion in vivo and stabilize the plaques. This is an interesting scientific topic and we will focus on that in our future work. The present study identified for the first time that FAK was involved in the regulation of MMP9 in macrophages, suggesting that FAK might be a potential therapeutic target for ACS.

Disclosures

Conflicts of interest: None.

References


