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Original Article

The effect of fetuin-A on the release of the inflammatory cytokine HMGB1 from a calcium phosphate crystal-stimulated human macrophage cell line in vitro

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Abstract: Objective: The purpose of this study was to investigate the effects of fetuin-A on the inflammatory response of a human macrophage cell line induced by calcium phosphate (CaP) crystals as a model for macrophage inflammation in nephrolithiasis. Methods: The macrophages were stimulated with 100 μg/ml CaP, or CaP and increasing concentrations of fetuin A (1-5 μg/ml) and cultured for 0, 6, 12, 18, 24, 30, 36, and 48 h before HMGB1 mRNA and protein levels were determined by real-time PCR, and Western blot analysis. IL-1β, IL-6, TNF-α, and MCP-1 levels were determined by ELISA. Results: Significant increases in HMGB1 mRNA levels were observed from 18 to 24h ($P < 0.01$), and after 12 h CaP-stimulation, HMGB1 gradually increased, reaching peak levels at 18 h. Whereas cytoplasmic HMGB1 increased gradually from 12 to 48 h after stimulation, nuclear HMGB1 decreased gradually from 18 to 24 h ($P < 0.01$), but increased from 30 to 48 h ($P < 0.01$). After macrophages were stimulated with CaP for 1 h, the contents of IL-1β, IL-6, TNF-α, and MCP-1 in cell culture supernatant were gradually increased and reached a peak after 4 h. HMGB1 mRNA and protein expression were lower in all fetuin-A-groups compared to the CaP group ($P < 0.05$). The contents of IL-1β, IL-6, TNF-α, and MCP-1 in the cell culture supernatant were all lower compared with the CaP group ($P < 0.05$). Conclusions: The expression of HMGB1, IL-1β, IL-6, TNF-α, and MCP-1 in macrophages can be induced by CaP. The release of HMGB1 lagged behind IL-1β, IL-6, TNF-α, MCP-1 release and protein levels translocated from the nucleus to the cytoplasm, to be finally released into the supernatant. Fetuin-A inhibited the synthesis and release of HMGB1, and the release of IL-1β, IL-6, TNF-α, and MCP-1 from macrophages induced by CaP in a concentration-dependent manner.

Keywords: Fetuin-A, HMGB1, macrophages, calcium phosphate crystals

Introduction

The vast majority of patients with urolithiasis have formed stones in which calcium oxalate (CaOx) is the dominating component. Calcium phosphate in the crystal phase (CaP) commonly accompanies CaOx, albeit usually only in small amounts. Various studies have shown that crystal-induced macrophage inflammation is involved in the etiology of nephrolithiasis [1-5]. The high-mobility group box 1 (HMGB1) protein is a nuclear DNA-binding protein. Activated macrophages and monocytes secrete HMGB1 as a cytokine mediator of inflammation. HMGB1 is an advanced stage inflammatory cytokine that leads to an amplification of the inflammation cascade by activation of macrophage cytokine release. It plays a key role in the onset and development of inflammation. Targeting HMGB1 may be beneficial in the treatment of calcium crystal-induced macrophage inflammation. Fetuin-A is a hepatocyte acidic glycoprotein with a molecular weight of 59 kDa that is present in blood serum. Recent reports show that fetuin-A has a powerful anti-inflammatory function through binding to its target HMGB1 [6, 7]. Therefore, to investigate the effect of fetuin-A on the synthesis and release

of HMGB1, IL-1β, IL-6, TNF-α, and MCP-1 from macrophages, the U937 human monocytes cell line is differentiated into macrophages and treated with fetuin-A in (CaP)-induced macrophage inflammation model for nephrolithiasis.

Materials and methods

Cell cultivation, differentiation, and experimental stimulation in a macrophage inflammation model

Macrophages were generated from the U937 human monocyte cell line using phorbol myristate acetate (PMA) for differentiation. U937 cells (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were maintained in a humidified incubator with 5% CO_2 at 37°C using RPMI 1640 (HyClone, GE Healthcare, Piscataway, NJ, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA.) in 25 cm^2 tissue culture flasks. The cell suspensions at a density of 1×10^6 cells/ml were treated with 100 ng/ml PMA (Fluka; Singapore) for 12 h (induction phase) as previously described [8]. After 12 h induction phase, the PMA-treated cells were vigorously washed with PBS twice to remove PMA and non-adherent cells, whereas the adherent cells were further maintained for 48 h (recovery phase). The macrophages were activated with 100 μg/ml CaP, and cultured for 0, 6, 12, 18, 24, 30, 36, and 48 h. After the total RNA of the cultured cells was extracted, HMGB1 mRNA was determined by real-time PCR and protein was determined by Western blot analysis in cell culture supernatants, cytoplasm, and nuclear fractions of macrophages. After the cells were cultured for 0 h, 1 h 2 h, and 4 h, the cell culture supernatants were collected, the levels of IL-1β, IL-6, TNF-α, and MCP-1 were determined by ELISA. To activate macrophages, cells were stimulated with 100 μg/ml CaP, 100 μg/ml CaP+1000 ng/ml fetuin-A, 100 μg/ml CaP+2500 ng/ml fetuin-A, 100 μg/ml CaP+5000 ng/ml fetuin-A, respectively, and cultured for 24 h before analysis was performed.

Real-time polymerase chain reaction (RT-PCR) for HMGB1 in macrophages

Primers for HMGB1 and GAPDH were designed by TaKaRa (Shiga, Japan) as follows: HMGB1 (Genbank NM-0021), 5'-GATCCCAATGCACCCAAGAG-3' (forward primer), 5'-TCGCAACATCACCAATGGAC-3' (reverse primer) amplifying a 109-bp product; GAPDH (Genbank NM-002046.3) 5'-GCACCGTCAAGGCTGAGAAC-3' (forward primer), 5'-TGGTGAAGACGCCAGTGGA-3' (reverse primer) amplifying a 138-bp product. The total RNA of macrophages was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was reverse transcribed to cDNA according to the manufacturer's protocol. GAPDH was used as an internal control. PCR was performed in a reaction mixture consisting of 2 μl cDNA, 10 μl of SYBR Premix Ex Taq II (2×), 0.8 μl each of the forward and reverse primer set for HMGB1, GAPDH, 0.4 μl ROX Reference Dye, and 6 μl RNAse free water. After an initial 30 s denaturation at 95°C, the thermal cycling comprised 40 cycles of denaturation at 95°C for 5 s and at 60°C for 34 s, and annealing and extension at 95°C for 15 s; 60°C for 1 min and 95°C for 15 s. After the end of the reaction, a melting curve analysis was performed to validate the specificity of the PCR products, and an amplification curve analysis validated the integrity of the PCR products.

Western blot analysis for HMGB1 in macrophages and cell culture supernatant

Cytoplasmic, nuclear, and cell culture supernatant protein were extracted and assayed according to the manufacturer's protocol (Nuclear and Cytoplasmic Protein Extraction Kit, Shanghai Biyuntian Biological Co, Ltd.). Protein samples (46 μg) were separated by SDS PAGE gels and electroblotted onto PVDF membranes. The membranes were incubated with monoclonal antibodies against HMGB1 at 1:5000 dilution and monoclonal antibody against GAPDH at 1:5000 dilution (Sigma-Aldrich, St. Louis, MO, USA). Blots were developed with ECL detection reagent (Millipore, Billerica, MA, USA) and exposed to Fuji medical X-ray film. The blots were analyzed using the GelDoc 2000 gel imaging analysis system and quantied using the Quantity One software (Bio-Rad, Hercules, CA, USA).

ELISA for IL-1β, IL-6, TNF-α, and MCP-1 in macrophage cell culture supernatants

The content of IL-1β, IL-6, TNF-α, and MCP-1 in the cell culture supernatant of macrophage cultures was determined by ELISA according to the manufacturer's instructions (Unitech BIO-Ltd. Hang Zhou, China).

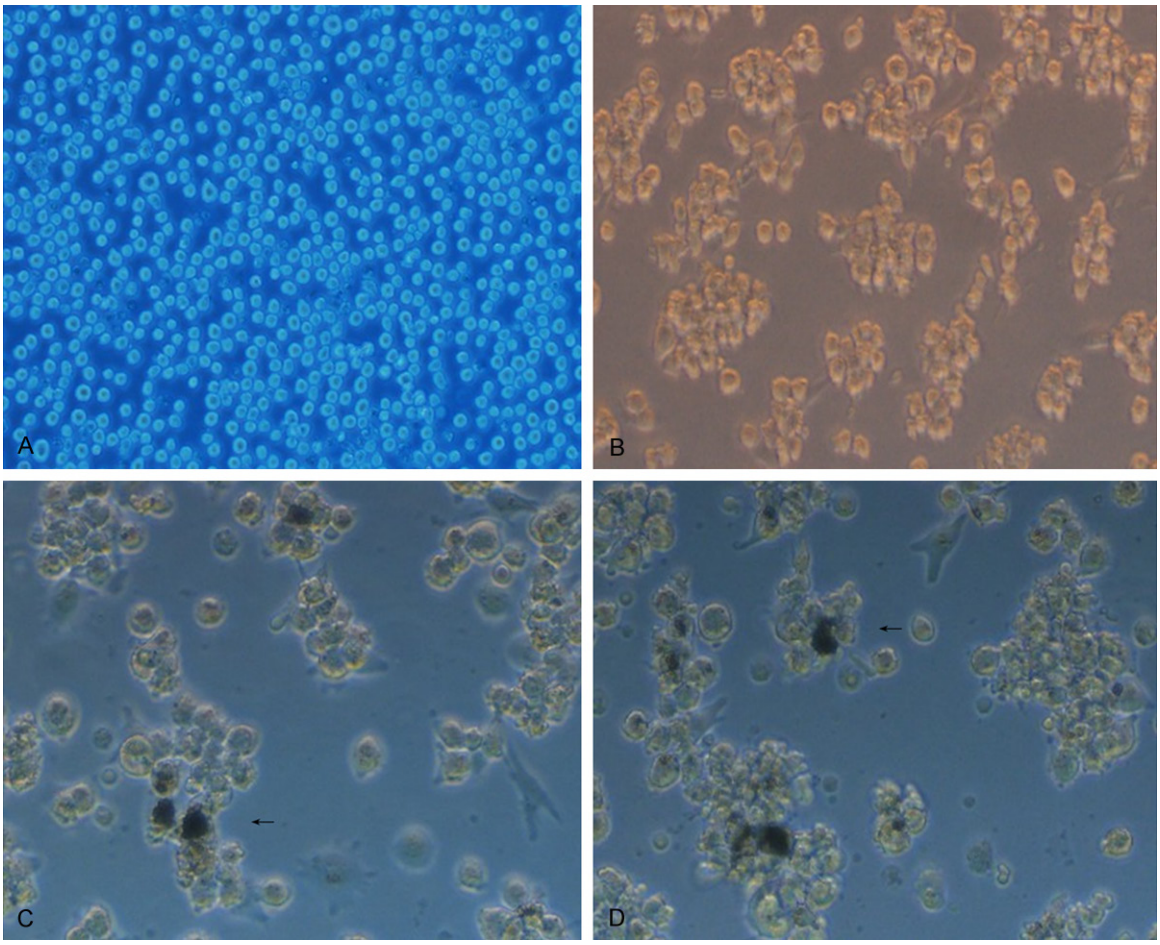

Figure 1. Treatment of U937 cells cells in culture with PMA, CaP, and CaP+Fetuin-A. Untreated cells in culture displayed a round, floating morphology (A) with morphological changes characteristic for macrophages after treatment with 100 ng/ml PMA for 12 h (B). Macrophages after treatment with CaP (C) or with CaP+Fetuin-A (D). Arrows point to typical CaP crystals (×200 magnification in A-D).

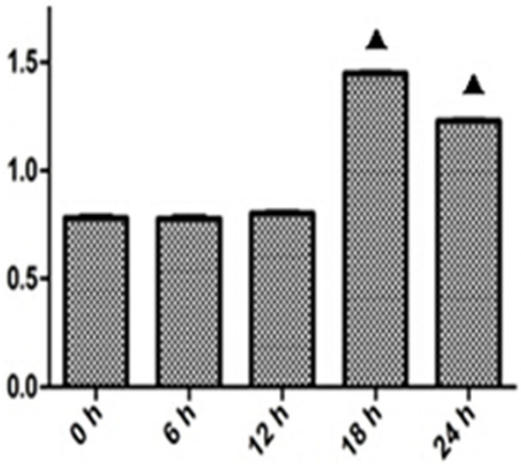

Figure 2. RT-PCR analysis of HMGB1 mRNA expression in cultured macrophages. Macrophages were stimulated with 100 μg/ml CaP for 0-24 h and HMGB1 expression levels were compared with GAPDH mRNA levels. ▲compared with 0 h, $P < 0.01$.

Statistical analysis

All statistical analysis was performed using the SPSS16.0 statistical analysis software (SPSS, Chicago, IL, USA), and the significance lever was set at $P < 0.05$ or $P < 0.01$. Statistical analysis was performed using the unpaired Student's t test. Those without significantly deviation from the normally distributed data were selected for One-way analysis of variance to determine significant differences among groups. Statistical analysis One-way analysis of variance and LSD-t test were used.

Results

Morphological changes in U937 monocyte culture after PMA differentiation and CaP incubation of macrophages

Cultured U937 monocyte cells were round, non-adherent cells floating in the medium

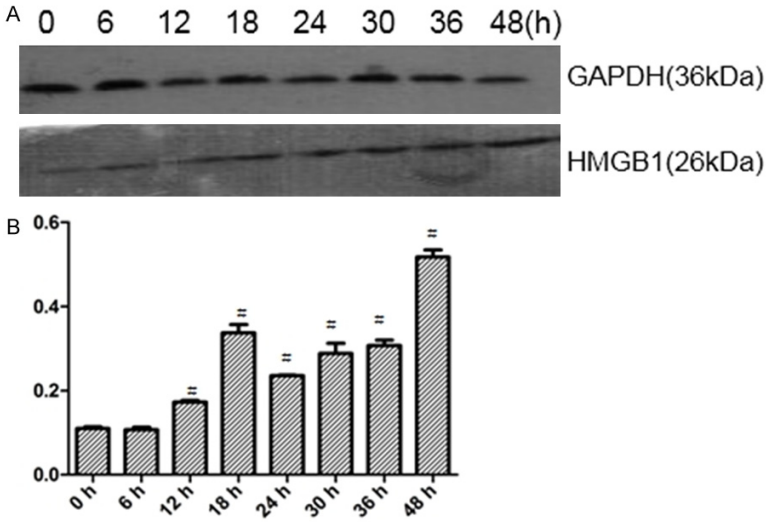

Figure 3. Western blot analysis of HMGB1 protein expression: (A) GAPDH and HMGB1 expression in culture supernatants of macrophage 0-48 h after stimulation with 100 μg/ml CaP crystals. (B) Quantification of HMGB1 protein expression shown in (A) normalized to the GAPDH control. #compared with 0 h, $P < 0.01$.

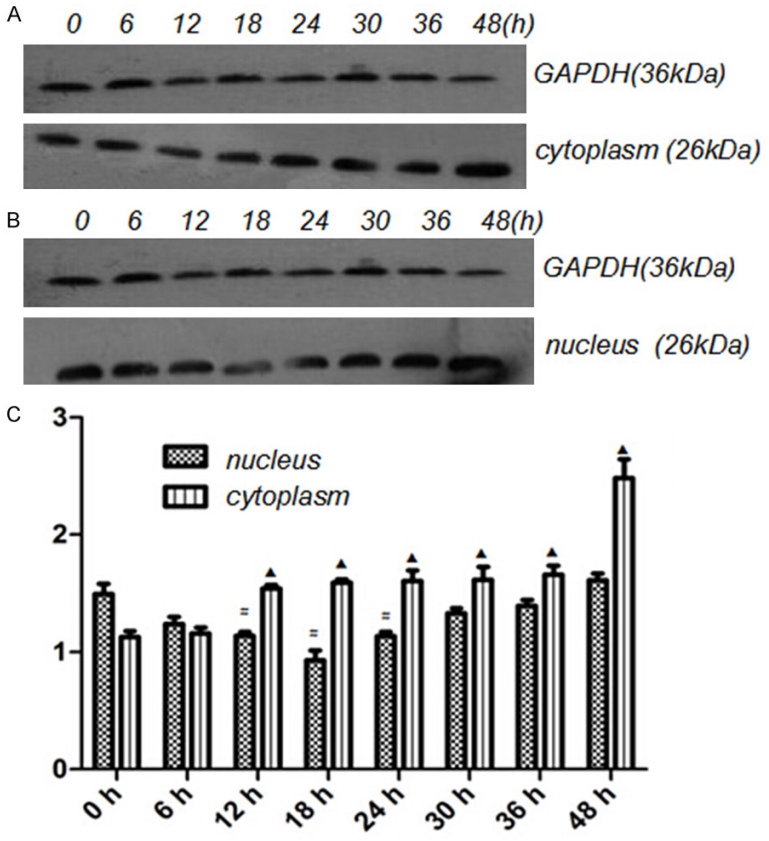

Figure 4. Western blot analysis of HMGB1 protein expression. HMGB1 expression in the (A) cytoplasm and the (B) nucleus of macrophages stimulated with 100 ug/ml CaP crystals. GAPDH protein expression served as control. (C) Quantification of HMGB1 protein expression in cytoplasm and nucleus of macrophages shown in the Western blots in (A and B) and normalized to GAPDH controls. #, compared with 0 h, $P < 0.01$. ▲, compared with 0 h, $P < 0.01$.

(**Figure 1A**). Treatment with PMA differentiated U937 cells into macrophages which adhered to the cell culture flask and showed characteristic pseudopodia (**Figure 1B**). When CaP was added to the macrophages in culture, CaP crystals had been surrounded by the macrophages. In CaP treated (**Figure 1C**) and CaP+Fetuin-A treated (**Figure 1D**) macrophages to a similar extend.

HMGB1 mRNA expression in macrophages stimulated by CaP crystals

HMGB1 mRNA expression in macrophages was analyzed by RT-PCR 0-24 h after treatment with CaP and compared with GAPDH mRNA controls. At 0-12 h CaP treatment, there was no obvious change in HMGB1 mRNA expression compared to GAPDH. After 18 h, HMGB1 mRNA expression of macrophages increased significantly and stayed elevated until 24 h after CaP treatment compared with 0-12 h ($P < 0.01$, **Figure 2**).

HMGB1 protein expression in culture supernatants of macrophages stimulated with CaP crystals

When HMGB1 protein levels in cell culture supernatants of macrophages which were stimulated with CaP crystals for 0-6 h were measured, only low levels of protein were detected (**Figure 3A**). However, starting at 12 h CaP incubation, HMGB1 levels increased significantly and reached a peak at 18 h as determined by quantification of Western blots (**Figure 3B**). After 48 h of incubation, a second HMGB1 peak appeared. HMGB1 levels showed significant eleva-

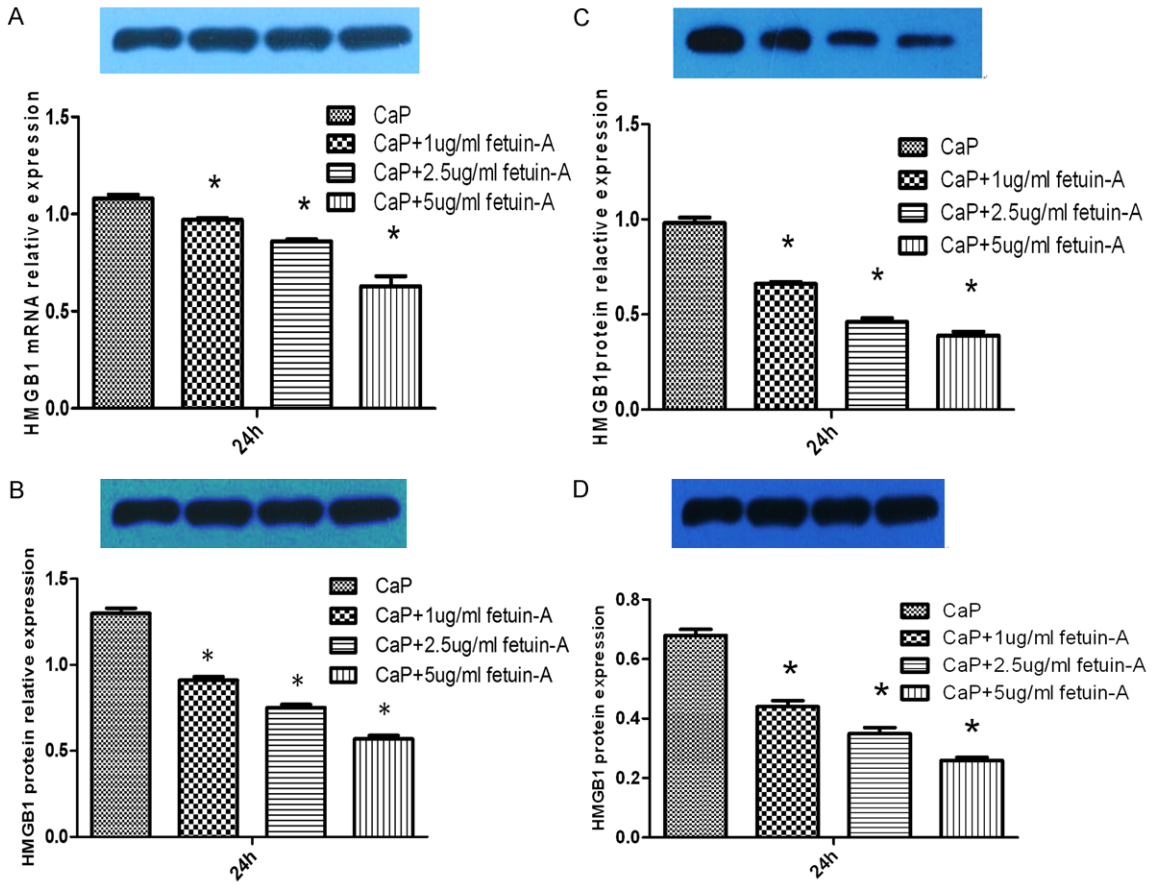

Figure 5. HMGB1 mRNA and protein expression in treated macrophages (A) RT-PCR reveals dose-dependent reduction of HMGB1 mRNA expression in the CaP+Fetuin-A groups compared with the CaP group (*P < 0.05). (B) HMGB1 protein expression in macrophages after stimulation with CaP and CaP+Fetuin-A for 24 h shows dose-dependent and significant reduction (A, CaP group; B, CaP +1 ug/ml Fetuin-A group; C, CaP +2.5 ug /ml Fetuin-A group; D, CaP +5 ug /ml Fetuin-A group; *P < 0.05). (C) HMGB1 protein expression in the cytoplasm of macrophages after stimulation with CaP and CaP+Fetuin-A for 24 h shows dose-dependent and significant reduction (A, CaP group; B, CaP +1 ug/ml Fetuin-A group; C, CaP +2.5 ug /ml Fetuin-A group; D, CaP +5 ug /ml Fetuin-A group; *P < 0.05). (D) HMGB1 protein expression in the cell culture supernatant of macrophages after stimulation with CaP and CaP+Fetuin-A for 24 h shows dose-dependent and significant reduction (A, CaP group; B, CaP +1 ug/ml Fetuin-A group; C, CaP +2.5 ug /ml Fetuin-A group; D, CaP +5 ug /ml Fetuin-A group; *P < 0.05). The relative HMGB1 protein expression was calculated using densitometry and normalized to GAPDH protein expression (B-D).

tion between 18-48 h of CaP crystal incubation (**Figure 3B**, P < 0.01).

HMGB1 protein expression in the cytoplasm and nucleus of CaP-stimulated macrophages

HMGB1 protein levels in the cytoplasm of macrophages were low in unstimulated macrophages, and when the macrophages were stimulated with CaP crystals for 0-6 h. After 12-48 h incubation, HMGB1 gradually increased in the cytoplasm and showed statistical significant increase compared to 0 h incubation (**Figure 4A**, P < 0.01). At 0 h, HMGB1 expression level in cell nucleus was relatively high, but decreased following CaP stimulation for 12-24 h (**Figure 4B**, P < 0.01). Between 30-48 h, the HMGB1 levels in the nucleus increased gradually and showed statistically significant differences compared to the protein levels at 12-24 h (**Figure 4C**, P < 0.01).

HMGB1 mRNA and protein expression in macrophages and culture supernatants after CaP and CaP+Fetuin-A stimulation for 24 h

HMGB1 mRNA expression was measured by RT-PCR in macrophages after stimulation with

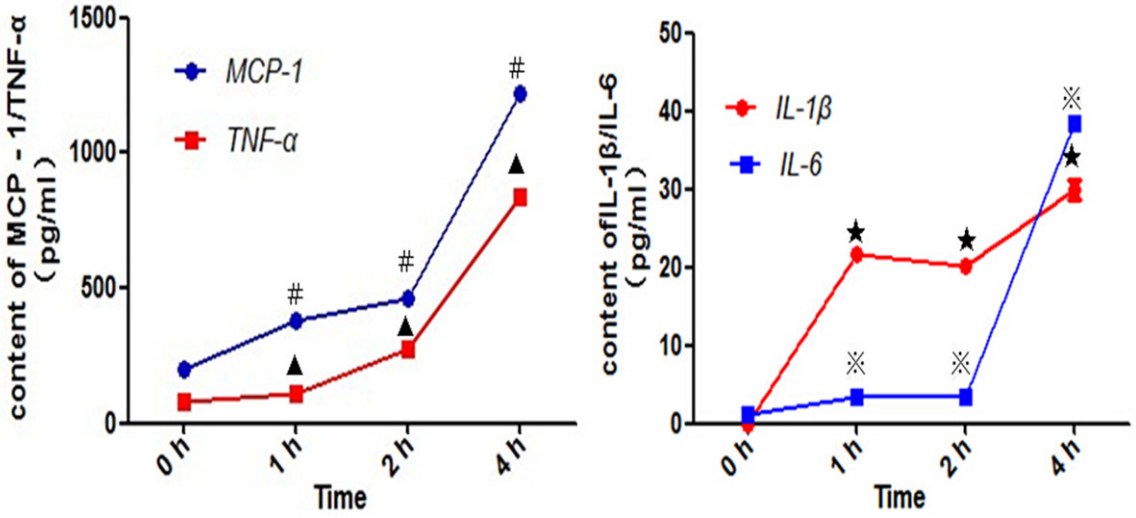

Figure 6. ELISA analysis of IL-1β, IL-6, TNF-α, and MCP-1 levels in culture supernatants of macrophages. The cytokines were detected at 0 h, 1 h, 2 h, and 4 h after stimulation with 100 ug/ml CaP. #, ★, ▲, ※, $P < 0.01$ compared with 0 h.

Table 1. ELISA analysis of IL-1β, IL-6, TNF-α, and MCP-1 levels in cell culture supernatants of macrophages stimulated with CaP and increasing concentrations of Fetuin-A

Group (n=4)	IL-1β (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)	MCP-1 (pg/ml)
CaP group	24.48±0.91	15.39±0.49	30.98±0.94	732.22±15.65
CaP+1 μg/ml Fetuin-A group	19.75±0.40*	13.72±0.52*	18.08±0.47*	504.09±17.67*
CaP+2.5 μg/ml Fetuin-A group	13.72±0.41*	8.88±0.51*	11.67±0.45*	459.83±19.10*
CaP+5 μg/ml Fetuin-A group	11.45±0.11*	6.23±0.19*	5.70±0.64*	361.89±18.90*

*$P < 0.05$, compared with the CaP group.

CaP and CaP+Fetuin-A for 24 h (**Figure 5A**). Compared with the CaP group, HMGB1 mRNA expression in the CaP+Fetuin-A groups was significantly lower (1 μg, 2.5 μg, and 5 μg, $P < 0.05$). The quantitative analysis of HMGB1 Western blot analysis in the whole cell fraction (**Figure 5B**), the cytoplasmic fraction (**Figure 5C**), and the cell culture supernatant (**Figure 5D**) of macrophages revealed that protein expression level significantly and dose-dependently decreased in all cellular fractions and in the cell culture supernatant of the CaP+Fetuin-A-treated group compared to the CaP group ($P < 0.05$).

Levels of inflammatory cytokines IL-1β, IL-6, TNF-α, and MCP-1 in the culture supernatant of macrophages stimulated with CaP and CaP+Fetuin-A

ELISA analysis of IL-1β, IL-6, TNF-α, and MCP-1 levels in cell culture supernatants of macrophages stimulated with CaP for 0-4 h showed significant increases over time in protein levels of the cytokines released into the supernatant of the activated macrophages (**Figure 6**). When increasing concentrations of Fetuin-A were applied to macrophage cultures treated with CaP for 4 h all cytokine levels decreased significantly compared with the CaP group (**Table 1**, *$P < 0.01$).

Discussion

Renal calculi are formed by a sophisticated mechanism. An increasing number of studies show that CaP crystal-induced inflammation plays an important role in renal calculus formation. The release of inflammatory factors from activated macrophages during CaP-crystal-induction can damage the renal interstitial, epithelial cells [1]. The High-mobility group box 1 (HMGB1) protein is a highly abundant nuclear protein that can be released from macrophages

or injured necrotic cells to promote the pathogenesis of inflammatory and autoimmune diseases once it is in an extracellular location. Extracellular HMGB1 mediates inflammatory processes via induction of cytokine production [9]. HMGB1 is composed of 215 amino acid residues, highly conserved in evolution, and involved in cell differentiation, DNA repair and recombination, regulation, and gene transcription regulation of steroid hormone activity [10]. Reports that CaP induces the release of HMGB1 from macrophages are still very rare. Wang reported that when macrophages were stimulated with lipopolysaccharide for 24 h, an endotoxin activating macrophages, no obvious change in HMGB1 mRNA gene expression was observed [11]. In our experiments, stimulation of macrophages with CaP crystals for 0-12 h led to no obvious change in HMGB1 mRNA expression, but after 18-24 h HMGB1 mRNA levels were significantly increased. When cultured macrophages were stimulated with CaP for 12 h, secreted HMGB1 protein was detectable in significant levels reaching a peak after 18 h, showing sustained release for 18-36 h and reaching another peak after 48 h. Before CaP stimulation, HMGB1 was mainly detected in the cell nucleus, and to a lesser extend in the cytoplasm. After 12-24 h CaP stimulation, HMGB1 in the cytoplasm increased gradually, while being reduced in the nucleus at the same time. At 30-48 h post-CaP-stimulation, HMGB1 in the cytoplasm stayed at elevated levels, while HMGB1 mRNA in the nucleus gradually increased. These results indicated that new HMGB1 synthesis occurred in macrophages at 24-48 h, and HMGB1 protein further translocated from the nucleus to the cytoplasm, and then was released into the culture supernatant. Therefore, the results show that CaP crystal-stimulation of macrophages for 0-24 h, HMGB1 produced in the nucleus, was gradually transferred to the cytoplasm, and then released. After stimulation for 18-24 h, HMGB1 mRNA gene expression in macrophages increased gradually, macrophages began to synthesize new HMGB1, which was again transferred to the cytoplasm and released into the culture medium. The coordination of new synthesis and release of HMGB1, lead to a gradual increase in HMGB1 expression in the nucleus after 30 h.

ELISA analysis of the inflammatory cytokines IL-1β, IL-6, TNF-α, and MCP-1 showed increase at 1 h after CaP-stimulation and reached a peak at 4 h. In contrast, HMGB1 in the culture supernatant increased at 12 h. This indicated that HMGB1 release lagged significantly behind that of IL-1β, IL-6, TNF-α, and MCP-1, which proved that HMGB1 was a “late” inflammatory factor.

Fetuin-A (also termed alpha-2-HS-glycoprotein for the human homologue), is a glycoprotein that is associated with bone formation, ectopic calcification and remodeling [12-15]. Fetuin-A is secreted from the liver, released into the blood where it inhibits arterial calcium deposition and acts as a potent inhibitor of ectopic mineralization [16]. Monomeric fetuin-A protein is a mineral carrier protein that binds to small clusters of calcium phosphate acting as a systemic inhibitor of pathological mineralization. It complements local mineralization inhibitors that act in a cell-restricted or tissue-restricted fashion [17]. In addition, more attention is paid to its anti-inflammatory features. Fetuin-A may contribute to the inactivation of macrophages [12]. Li et al. previously showed that fetuin-A plays a protective role in systemic inflammation by activating HMGB1 synthesis [18]. Our results show that HMGB1 mRNA and protein expression in macrophages treated with increasing levels of fetuin-A were lower compared with CaP group alone. Similarly, the inflammatory cytokines IL-β, IL-6, TNF-α, and MCP-1 were all reduced in the cell culture supernatant. These results indicate that fetuin-A may inhibit the expression of HMGB1 mRNA, inhibit the protein synthesis, transport, and release of HMGB1 and of IL-1β, IL-6, TNF-α, and MCP-1 following CaP-stimulation of macrophages. Previous studies have showed that the anti-inflammatory target for fetuin-A is HMGB1. Wang et al. found that exogenous fetuin-A may inhibit the release of HMGB1 in the mouse ischemic brain, and inflammation which limited the cerebral ischemic infarct zone [6]. Thus, the anti-inflammatory function of fetuin-A is significant and the anti-inflammatory target may be HMGB1. The results of the present study are consistent with previous reports. Stejskal et al. found that patients with urolithiasis had lower urine fetuin-A levels compared with controls [19]. Our previous study showed that the urinary and renal fetuin-A protein level decreased in patients with nephrolithiasis [20]. Fetuin-A deficiency dramatically increased the CaP-crystal-induced

inflammation response in the kidney, which may lead to kidney stone formation and recurrence.

In summary, our results indicate that fetuin-A may block the pro-inflammatory cytokine response network and inhibit the development of an inflammatory response in macrophages caused by CaP crystals. Therefore, fetuin-A might be a protective agent for nephrolithiasis at least partly through inhibiting inflammatory response induced by CaP crystals. However, the mechanisms underlying fetuin-A-mediated suppression of HMGB1 release may be complex. Binding of fetuin-A to CaP and thereby reducing the formation of CaP crystals and the inflammatory response may act in concert with the observed effect on expression and release of HMGB1 and inflammatory cytokines. Collectively, these data further establish HMGB1 as a critical "late" mediator of inflammation with a wider therapeutic window for nephrolithiasis. Supplementation with exogenous fetuin-A may be useful for reducing HMGB1 release by macrophages. Thus, fetuin-A may play an important protective role in CaP-induced inflammation leading to nephrolithiasis.

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Disclosure of conflict of interest

None.

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