主論文の要旨

Excessive salt intake increases peritoneal solute transport rate via local tonicity-responsive enhancer binding protein in subtotal nephrectomized mice

（腎不全マウスにおいて高塩分食は浸透圧応答性エンハンサー結合タンパク質によって腹膜透過性を増加させる）

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【Introduction】

High baseline peritoneal solute transport rate (PSTR), assessed as the dialysate-plasma ratio of creatinine (D/P Cr) within 6 months after PD initiation, was found to be related to mortality and technique failure of peritoneal dialysis (PD). According to our previous pathological study, a positive relationship existed between baseline PSTR and the number of blood vessels, macrophages and IL-6-positive cells in the uremic peritoneum. However, there still lacks studies about the causes of intraperitoneal inflammation before PDF exposure. Recent studies showed that sodium could be stored in the skin or muscle interstitium without proportionate water retention, and chronic kidney disease (CKD) patients on hemodialysis tend to have higher levels of such storage than the normal population. Furthermore, our recent work demonstrated macrophage infiltration and local inflammatory changes in the peritoneal wall and heart of mice with salt loading and subtotal nephrectomy (Nx), along with higher accumulation of tissue sodium.

The present study investigated the hypothesis that high salt intake under uremic condition could further induce chronic inflammation and angiogenesis, leading to functional and pathological changes to the peritoneum without exposure to PDF.

【Materials and Methods】

Mouse model

Eight- to 10-week old male 129x1SvJJmc mice were randomly divided into groups which underwent subtotal nephrectomy or sham operation and then were provided with tap water or 1% sodium chloride (NaCl)-containing water respectively for 8 weeks. Then a 4.25% glucose-based peritoneal equilibration test (PET) was performed. Dialysate and blood samples were collected. Mice were then euthanized and parietal peritoneum and diaphragm tissue samples were collected for further analyses.

Blockade of IL-6 signaling

Rat anti-mouse monoclonal IL-6 receptor antibody (MR16-1, rat IgG1) provided by Chugai Pharmaceutical was used to neutralize IL-6 signaling.

Cell Culture

Human mesothelial cell line Met-5A was used in the study. Medium was changed for serum-free medium 24 h before changing to medium containing 40 mM of additional NaCl. The supernatant and cell lysate were harvested after 24 h after stimulation with hypertonic medium. Small interfering RNA (siRNA) for tonicity-responsive enhancer binding protein (TonEBP) and non-targeting control siRNA were transfected into Met-5A cells using Lipofectamine RNAiMAX Reagent.

【Results】

Evaluated by a 4.25% PET, D/P Cr showed a rise in Nx mice with tap water (Nx+water)

compared to sham-operated mice with tap water (Sham+water), whereas Nx mice with 1% NaCl in drinking water (Nx+salt) exhibited a significantly greater increase. The ratio of dialysate glucose at 2 h of dwell time to dialysis glucose at 0 h of dwell time (D/D0 glucose) showed a decrease in Nx+salt mice. Furthermore, protein concentration was significantly increased in the dialysate of Nx+salt mice (**Figure 1A, B**). Altogether, these results suggested that excessive salt intake under uremic conditions could lead to faster peritoneal transport status without exposure to PDF.

Significant elevation was observed in the staining of CD31-positive blood vessels and CD68-positive macrophages in the parietal peritoneum of Nx+salt mice. VEGF-A, as the main pro-angiogenetic factor, demonstrated marked staining in the mesothelial layer of the parietal peritoneum of Nx+salt mice, while no significant expression was found in other groups. Higher concentrations of VEGF-A as well as monocyte chemoattractant protein (MCP)-1 in the dialysate were also detected in Nx+salt mice (**Figure 1C, D**).

We detected that the concentration of IL-6 in dialysate demonstrated a marked elevation in Nx+salt group ($p<0.05$ compared to Nx+water) although serum IL-6 levels showed no significant difference between Nx+salt and Nx+water mice (**Figure 2A**). To further investigate the role of IL-6 in high salt intake-induced increases in peritoneal transport, we examined whether blocking IL-6 signaling by a monoclonal rat anti-mouse IL-6R antibody, MR16-1, could improve peritoneal function in Nx+salt mice. Administration of MR16-1 significantly decreased the PSTR and attenuated dialysate protein leakage (**Figure 2B**). In addition, a prominent decrease in CD31-positive vessel density and CD68-positive macrophages was seen in the parietal peritoneum of MR16-1-treated mice. Meanwhile, VEGF-A and MCP-1 concentrations showed significant decreases in the dialysate (**Figure 2C, D**), suggesting that angiogenesis and macrophage recruitment were, at least partially, attributable to IL-6 signaling.

We further examined the mechanisms of peritoneal changes induced by salt loading in uremic mice. As we previously reported, increased sodium accumulation was found in the abdominal walls of subtotal nephrectomized mice with salt loading, leading to local hypertonic stimuli. As TonEBP is the only transcription factor known to respond to high tonicity to date, we then examined TonEBP expression in peritoneum and observed a significant higher transcript level in Nx+salt mice (**Figure 3A**). We also examined the role of TonEBP in human mesothelial cell line Met-5A, which were incubated with an additional 40 mM of NaCl in the medium up to 24 h and an increase of TonEBP mRNA after high salt incubation was observed. Meanwhile, significant elevation of IL-6, MCP-1, VEGF-A and VEGF-C mRNA and protein expression was observed, which was decreased by knockdown of TonEBP by siRNA (**Figure 3B, C**).

To further examine whether peritoneal function could be improved by removal of the high-salt diet, we performed another animal experiment in which a group of mice was

removed from 1% NaCl-containing water from midway. Compared with Nx mice ingesting 1% NaCl-containing water for 8 weeks (Salt 8w), Nx mice ingesting 1% NaCl-containing water for 4 weeks and tap water for 4 weeks (Salt 4w+water 4w) showed a lower peritoneal TonEBP level as well as lower D/P Cr and higher D/P glucose, suggesting a reversal of peritoneal function by abandoning high salt intake in uremic mice (**Figure 3D**).

【Discussion】

Differences in baseline PSTR between patients should be noticed from the beginning of PD therapy. Here, using in vivo and in vitro experiments, we demonstrated that high tonicity induced the expression of transcription factor TonEBP as well as IL-6 and VEGF-A in mesothelial cells and high salt intake under uremic conditions led to higher peritoneal TonEBP expression and higher solute transport rate (**Figure 4**).

Nx+salt mice showed increased peritoneal blood capillary density as well as dialysate VEGF-A level. Besides strong pro-angiogenetic capacity, VEGF-A could also promote vascular leakage via increasing diffusion, interendothelial junctions and the formation of small pores on endothelial cell cytoplasm. Elevation of VEGF-A, we suppose, was partially responsible for both enhanced PSTR and protein leakage in Nx+salt mice. The blood endothelial surface layer, which is abundant in glycosaminoglycans, has also been considered to play a role in sodium homeostasis, so further investigation about effects of it on vascular permeability is also needed.

IL-6 could increase endothelial permeability by changing cell shape and readjusting actin filaments. IL-6 trans-signaling, which is mediated by soluble IL-6 receptor α (sIL-6Rα), contributes to VEGF-A and MCP-1 production in various cells, including mesothelial cells. Notably, MR16-1 treatment decreased IL-6 signaling and improved peritoneal transport function in Nx+salt mice, indicating a significant role of IL-6 during the progression of peritoneal inflammation and inflammation-related angiogenesis, suggesting that IL-6 accounted for peritoneal vascular leakage as well as part of VEGF-A and MCP-1 production.

Commonly, salt restriction has been a crucial approach for CKD patients due to the widely known benefits in terms of blood pressure control. Recently it is considered that renal effects, systemic vascular resistance and the vascular endothelial surface layer are all involved in the physiology of salt-sensitive hypertension, which could be disturbed under conditions of uremia. Therefore, patients with CKD are more salt-sensitive than the normal population. Skin or muscle sodium contents and TonEBP expression could offer prospective indicators of baseline peritoneal transport function, and should also be investigated after PD induction in future projects.

The present study found an obviously increased level of peritoneal TonEBP expression in Nx+salt mice, but not in Sham+salt mice, suggesting a local hypertonic status.

Hypertonicity-sensing transcription factor TonEBP is abundant in the kidney and protects renal cells from high osmolality. Moreover, increased extrarenal TonEBP level was found to be related to skin lymphangiogenesis, cardiac inflammation, rheumatoid arthritis and cancer. TonEBP is known to enhance the activity of transcription factor nuclear factor-κB and promote expression of pro-inflammatory factors. The present study demonstrated that TonEBP was involved in regulating IL-6, MCP-1, VEGF-A and VEGF-C expression under hypertonicity.

【Conclusion】

Excessive salt intake under uremic conditions induced TonEBP upregulation and caused angiogenetic and inflammatory changes in the peritoneum, leading to dysfunction of peritoneal transport. Our experimental data provided a novel view that salt restriction in CKD patients might preserve peritoneal transport function before the initiation of PD.