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Disintegration of colonic epithelial tight junction in uremia: a likely cause of CKD-associated inflammation

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Abstract

Background. Inflammation is a constant feature and a major mediator of the progression of chronic kidney disease (CKD) and its numerous complications. There is increasing evidence pointing to the impairment of intestinal barrier function and its contribution to the prevailing inflammation in advanced CKD. Under normal condition, the intestinal epithelium and its apical tight junction prevent entry of the luminal microorganisms, harmful microbial by-products and other noxious contents in the host's internal milieu. This study was designed to test the hypothesis that impaired intestinal barrier function in uremia must be due to disruption of the intestinal tight junction complex.

Methods. Sprague–Dawley (SD) rats were randomized to undergo 5/6 nephrectomy (CKD) or sham-operation (control) and observed for 8 weeks. In a separate experiment, SD rats were rendered uremic by addition of 0.7% adenine to their food for 2 weeks and observed for an additional 2 weeks. Rats consuming a regular diet served as controls. The animals were then euthanized and their colons were removed and processed for expression of the key constituents of the tight junction complex using real-time polymerase chain reaction, western blot analysis and immunohistological examinations.

Results. The CKD groups showed elevated plasma urea and creatinine, reduced creatinine clearance, thickened colonic wall and heavy infiltration of mononuclear leukocytes in the lamina propria. This was associated with marked reductions in protein expressions of claudin-1 (70–90%), occludin (50–70%) and ZO-1 (80–90%) in the colonic mucosa in both CKD models compared with the corresponding controls. The reduction in the abundance of the given proteins was confirmed by immunohistological examinations. In contrast, messenger RNA abundance of occludin, claudin-1 and ZO-1 was either unchanged or elevated pointing to the post-transcriptional/post-translational modification as a cause of the observed depletion of the tight junction proteins.

Conclusion. The study revealed, for the first time, that uremia results in depletion of the key protein constituents of the colonic tight junction, a phenomenon which can account for the impaired intestinal barrier function and contribute to the systemic inflammation in CKD.

Keywords: claudin; end-stage renal disease; gastrointestinal tract; intestinal barrier defect; occludin

Introduction

Systemic inflammation and its companion oxidative stress are constant features and major mediators of cardiovascular disease, cachexia, anemia and numerous other morbidities in patients with advanced chronic kidney disease (CKD) [1–5]. Inflammation in this population is frequently associated with endotoxemia in the absence of clinical infection [6–8]. One of the most likely sources of endotoxemia in the infection-free patients with advanced CKD is the microbial flora of the gastrointestinal tract. However, passage of endotoxin from the intestinal lumen to the circulation can occur only if the intestinal mucosal barrier function is impaired. In fact increasing evidence has emerged pointing to the impairment of the intestinal barrier function and its potential contribution to the prevailing inflammation in uremic animals and humans [9]. This supposition is based on the following observations: (i) earlier studies by Magnusson *et al.* [10, 11] demonstrated increased intestinal permeability to large molecular weight polyethylene glycols in uremic humans and animals, (ii) studies by de Almeida Duarte *et al.* [12] demonstrating increased penetration of bacteria across the intestinal wall and their detection in the mesenteric lymph nodes in rats with uremia induced by subtotal nephrectomy, (iii) prevalence of endotoxemia in patients with end-stage renal disease (ESRD) [6, 8] and (iv) presence of histological evidence of chronic inflammation throughout the gastrointestinal tract including esophagitis, gastritis, duodenitis, enteritis and colitis in chronic hemodialysis patients [13].

The primary functions of the intestinal mucosa are absorption of nutrients, secretion of waste products and serving as a barrier to prevent absorption of waste

products and entry of luminal microorganisms and their harmful by-products in the host's internal milieu. The intestinal epithelial barrier consists of the apical plasma membrane of the enterocytes which regulates passive and active transcellular transport of solutes (usually via specific transport channels) and the apical junctional complex which forms the barrier against paracellular permeation of luminal substances [14, 15]. The apical junctional complex includes tight junction and the subjacent adherens junction of which the tight junction is the most luminal component. The tight junction consists of the following transmembrane and cytosolic components [14–16]: (i) the adhesive transmembrane proteins which form the barrier to diffusion of fluids and solutes by linking the plasma membranes of the adjacent cells, (ii) the actin-binding cytosolic tight junction proteins which regulate the organization and positioning of the apical junction complex [14] and (iii) the peri-junctional ring of actin and myosin which regulate paracellular permeability by modulating the structure and function of the tight junction. The tight junction's transmembrane proteins consist of occludin, claudin family of proteins and junctional adhesion molecule-A. The cytosolic plaque proteins include members of the zonula occludens (ZO) protein family of which ZO-1 is the key members. Through its multiple protein interaction domains, ZO-1 binds to the transmembrane proteins and to the underlying peri-junctional actomyosin ring and as such serves an essential role in the tight junction assembly and function [14]. Normally, intestinal epithelial tight junction serves as an effective barrier against penetration of noxious substances and antigens including bacteria, bacterial toxins, bacterial by-products, digestive enzymes and degraded food products contained in the gastrointestinal lumen.

In view of the emerging evidence of increased intestinal permeability in uremia and the critical role of the tight junction in the mucosal barrier function, we hypothesized that uremia may impair the integrity of the intestinal tight junction complex. The present study was designed to test this hypothesis.

Materials and methods

Animals

Male Sprague–Dawley (SD) rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). The rats were randomized to undergo sham operation (control) or 5/6 nephrectomy (CKD) by surgical resection using a dorsal incision, as described previously [17] and observed for 8 weeks. The surgical procedures were carried out under general anesthesia using intraperitoneal (IP) injection of Ketamine/Xylazine. Strict hemostasis and aseptic techniques were observed. In a separate experiment, SD rats were rendered uremic by addition of 0.7% adenine to their food for 2 weeks and observed for an additional 2 weeks. Rats consuming a regular diet served as controls. The animals were housed in a temperature-controlled facility with 12-h light/dark cycles. At the conclusion of the given observation periods, animals were placed in metabolic cages for a 24-h urine collection. They were then anesthetized (Ketamine/Xylazine IP) and euthanized by exsanguinations using cardiac puncture. The ascending and descending colon were harvested and processed for expression of the key constituents of the tight junction proteins using real-time polymerase chain reaction (RT-PCR), western blot analysis and histological and immunohistological examinations. All experiments were approved by the University of California, Irvine Institutional Committee for the Use and Care of Experimental Animals.

Western blot analyses

The tissues were homogenized on ice in modified radioimmunoprecipitation assay lysis buffer containing 25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraaccetic acid, 1% NP-40, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride and Protease Inhibitor Cocktail (Sigma–Aldrich, St louis, MO). Protein concentration in the tissue homogenates was determined by bovine serum albumin assay kit (Pierce Rockford, IL) and 60 μg of total protein from each sample were fractionated on 4–12% Bis-Tris gradient gel (Invitrogen, Carlsbad, CA) at 120 V for 2 h and transferred to a nitrocellulose membrane. The membrane was then incubated with rabbit anti-claudin-1 or rabbit anti-occludin and mouse anti-ZO-1 (Invitrogen) at 1:250 dilutions or mouse anti-Phospho-Myosin Light Chain 2 (Ser19) at 1:1000 dilution (Cell Signaling Technology, Danvers, MA) and anti-actin antibodies (Sigma–Aldrich) at 1:10 000 dilutions overnight. The appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma–Aldrich) were used at a 1:5000 dilution. The membrane was visualized with SuperSignal West Pico (Pierce) and developed by autoluminography.

Histological and immunohistochemical procedures

Tissue sections used for the histological examination were deparaffinized with xylene, re-hydrated with graded alcohol and stained with hematoxylin and eosin. For immunohistochemical analysis, paraffin sections were deparaffinized with xylene, and antigens were unmasked using sodium citrate buffer in a high-power pressure cooker for 10 min and then cooled down to room temperature. Endogenous peroxidase activity was removed using 3% hydrogen peroxide in water and blocked with protein block serum-free (Dako North America, Inc., Carpinteria, CA). The sections were incubated overnight at 4°C with primary antibodies [1:50 or 1:80 rabbit anti-claudin-1 or rabbit anti-occludin and mouse anti-ZO-1 (Invitrogen)], antibody binding was amplified using ImmPRESS™ REAGENT anti-rabbit Ig or anti mouse kit (Vector laboratories, Inc., Burlingame, CA) and the complex visualized using diaminobenzidine (DAB). Nuclei were lightly stained with Mayer's hematoxylin.

Real-time polymerase chain reaction

RT-PCR was performed using a Bio-Rad iCycler and their SYBR green PCR mix (Bio-Rad, Hercules, CA). Total RNA (5 μg) was isolated from the ascending and descending colon of control and chronic renal failure (CRF) rats and primed with oligo-dT primers to synthesize first strand complementary DNA (RT-PCR kit; Invitrogen). To amplify the coding region of rat occludin-1, ZO-1, claudin-1 and β-actin, we used gene-specific primers for rat occludin (forward: 5′-AGTACATGGC-TGCTG CTGATG-3′, reverse: 5′-CCCACCATCCTCTTGATGTGT-3′); Z01 (forward: 5′-AGCGAAGCCACCTGAAGATA-3′, reverse: 5′-GATGGC-CAGCAGGAATATGT-3′), claudin-1 (forward: 5′-CTGGGAGGTGC CCTACTTT-3′, reverse: 5′-CCGCTGTCACACGTA GTCTT-3′) and β-actin (forward: 5′-GTCAGGTCACTATCGGC-3′, reverse: 5′-CATG-GATGCCACAGGATTCC-3′). Qualitative real-time PCR conditions were followed as described by vendor. Data were normalized to β-actin and then quantified using a relative relationship method supplied by the iCycles manufacture (Bio-Rad) and as described previously [18].

Monocyte chemo attractant protein-1 (MCP-1) assay

Plasma monocyte chemo attractant protein-1 (MCP-1) was measured by enzyme-linked immunosorbent assay method using a kit purchased from Thermo Scientific and Life Science Research Products (Rockford, IL).

Data analysis

Student's *t*-test was used in statistical evaluation of the data which are shown as mean ± SD, unless otherwise specified. P-values <0.05 were considered significant.

Results

General data

Data are summarized in Table 1. As expected, the animals in both CKD groups exhibited significant increase in plasma urea and creatinine concentrations and a

significant fall in creatinine clearance. This was associated with significant elevation of arterial pressure in the 5/6 nephrectomized group but less so in the animals with adenine-induced CKD. Urine protein excretion was significantly increased in the rats with 5/6 nephrectomy-induced CKD but only minimally so in rats with adenine-induced CKD. Urine output was increased in both CKD groups but much more so in the group with adenine-induced CKD reflecting severe impairment of urinary concentrating ability. Differences observed in the urinary protein excretion and the magnitude of polyuria between the two CKD groups are consistent with the tubulointerstitial nature of renal injury in adenine-treated animals.

Histological findings

Histological examination of colonic tissues revealed increased wall thickness and accumulation of mononuclear leukocytes in the lamina propria and microvilli in the CRF animals (Figure 1). These findings are consistent with the results of the earlier studies in patients with ESRD [13]

Table 1. Body weight (BW), tail arterial pressure (BP), hematocrit (Hct), serum creatinine (SCr) and urea (SUrea) and urine protein excretion (U. protein) in the rats with CKD induced by consumption of adenine-containing food (upper panel) or subtotal nephrectomy (lower panel) and the corresponding control groups

Adenine-induced CKD and the corresponding control (CTL) groups

ID	BW (g)	BP (mmHg)	Hct (%) (%)	SCr (mg/dL)	SUrea (mg/dL)	Ccr (mL/min/kg)	U_Protein (mg/mg)
CTL	345 + 5.6	124 + 3.3	46 + 0.7	0.29 + 0.03	44.6 + 4.2	7.5 + 0.7	6.2 + 0.4
CRF	298 + 3.2*	147 + 3.7*	31 + 0.8*	1.03 + 0.17*	96.5 + 7*	1.8 + 0.2*	8.1 + 0.5*

Subtotal nephrectomy-induced CKD and the corresponding control (CTL) groups

ID	BW (g)	BP (mmHg)	Hct (%) (%)	SCr (mg/dL)	SUrea (mg/dL)	Ccr (mL/min/kg)	U_Protein (mg/mg)
CTL	407 + 5.9	120 + 2.1	48 + 1.2	0.61 + 0.2	48 + 3.3	8.8 + 0.05	7.4 + 0.5
CRF	374 + 4.4	155 + 2.5*	35 + 0.7*	1.14 + 0.2*	93 + 7.4*	3.4 + 0.03*	81.5 + 5.6*

*P < 0.05 compared with CTL.

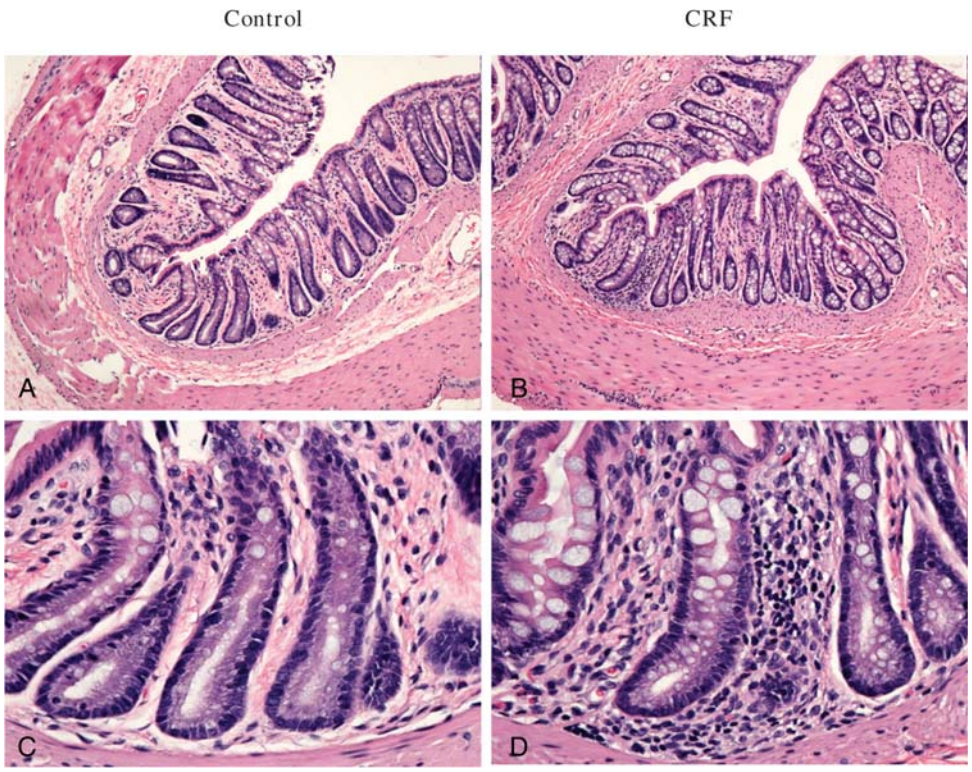

Fig. 1. Representative photomicrographs of the H&E-stained sections of colonic tissue in a rat with CKD induced by subtotal nephrectomy and a sham-operated control rat. The study revealed thickening of the wall and increased accumulation of mononuclear leukocytes in the CKD versus control rats. Original magnifications: ×10 upper panel and ×40 lower panel. Data are from at least six rats used in each group.

The tight junction protein data

Data are illustrated in Figures 2 and 3. The CRF group showed a marked reduction in protein expressions of claudin-1 in the ascending ($P < 0.01$) and descending ($P < 0.05$) colon. Similarly, protein abundance of occludin was significantly ($P < 0.05$) diminished in the ascending and descending colon of the CKD rats compared with the corresponding control animals. The reduction in expression of claudin-1 and occludin, the main transcellular constituents of tight junction, was accompanied by marked ($P < 0.001$) reduction of the main cytosolic plaque protein, ZO-1 in the ascending and descending colon in the CKD animals as compared to those found in the control rats. The reduction in protein abundance of the given molecules was confirmed by immunohistological examinations (Figure 3). Depletion of the transcellular and intracellular components of colonic epithelial tight junction was accompanied by diminished abundance of phosphorylated myosin light chain (Figure 4).

The RT-PCR data

Data are shown in Figure 5. The steady state messenger RNA (mRNA) expression of occludin, claudin-1 and ZO-1 in ascending and descending colon was examined by real-time PCR using gene-specific primers to amplify the coding region of rat occludin, ZO-1 and claudin-1. Data were normalized relative to the housekeeping gene β-actin. The results showed a significant increase in occludin ($P < 0.05$) and ZO-1 ($P<0.01$) mRNA expressions in the CKD rats' ascending colon compared to controls. In contrast, no significant difference was observed in either occludin or ZO-1 mRNA expression in the descending colon between the CKD and control groups. No significant difference was found in claudin-1 mRNA expression in either the ascending or descending colon between the CKD and control groups.

Plasma MCP-1 data

The CKD animals had a significant increase in plasma MCP-1 concentration denoting presence of systemic inflammation (Figure 6).

Discussion

Under normal condition, the intestinal epithelial tight junction prevents paracellular penetration of bacteria and their toxic by-products, digestive enzymes, and degraded food material [14, 15]. However, in certain

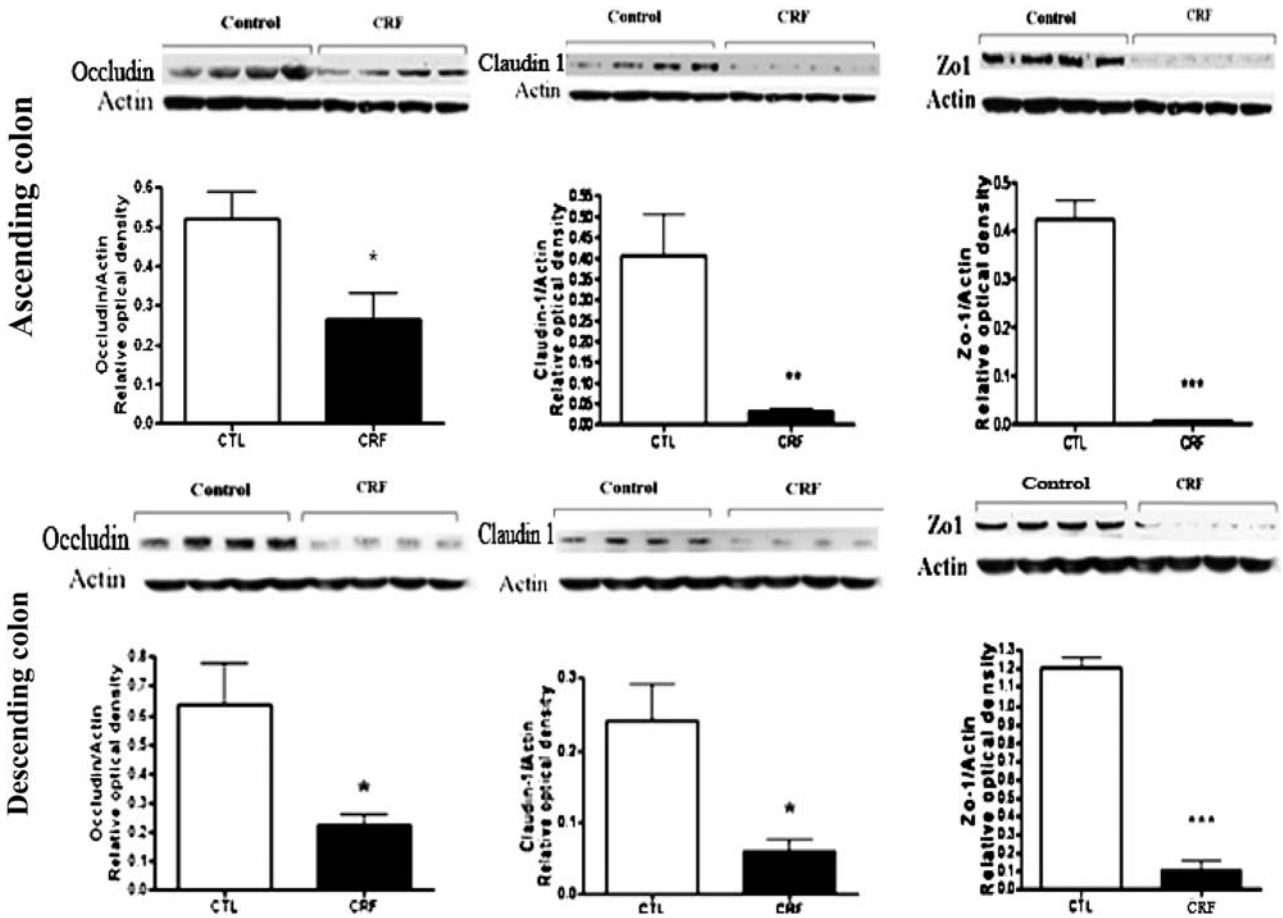

Fig. 2. Representative western blots and group data depicting protein abundance of occludin, claudin 1 and ZO-1 in the ascending and descending colonic tissues of the CKD and control (CTL) groups. *$P < 0.05$, **$P < 0.001$, ***$P < 0.001$, $N = 6$ animals in each group.

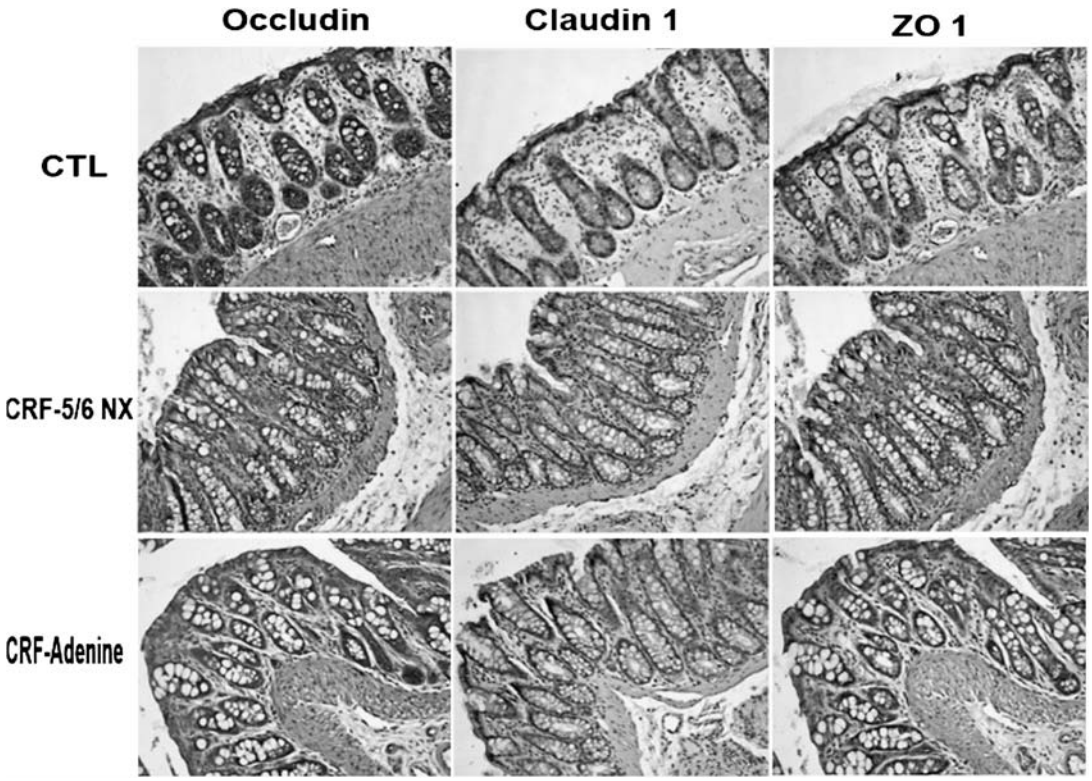

Fig. 3. Representative photomicrographs depicting occludin, claudin-1 and ZO-1 immunostaining in the ascending and descending colon of a rat with CKD induced by subtotal nephrectomy or injection of a diet containing 0.7% adenine and a sham-operated control rat.

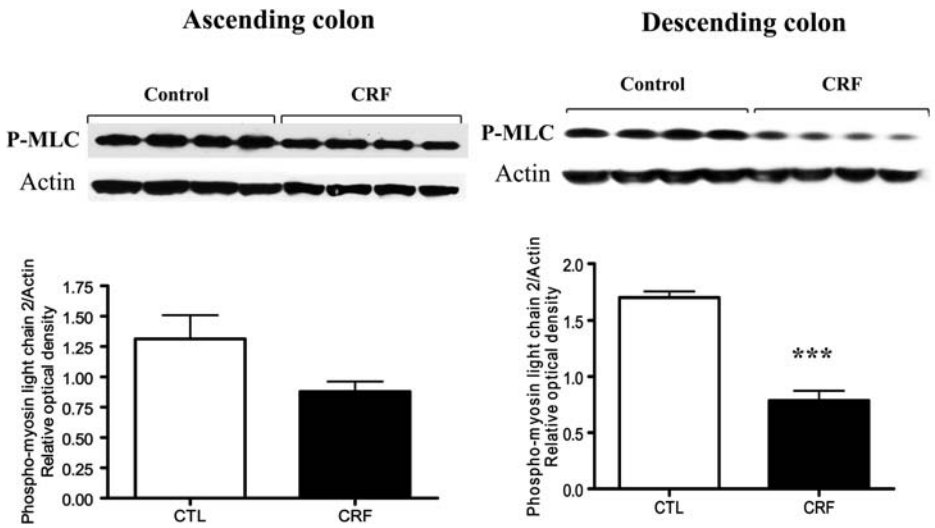

Fig. 4. Representative western blots and group data depicting the abundance of phosphorylated myosin light chain (P-MLC) in the colonic tissues of the CKD and control (CTL) groups. ***$P < 0.001$, $N = 6$ animals in each group.

pathological conditions, such as in ulcerative colitis, Crohn's disease, heat stroke, alcoholic hepatitis and *Escherichia coli*, *Clostridium difficile* and *Vibrio cholera* infections, the intestinal tight junction barrier is impaired allowing permeation of luminal antigens and pro-inflammatory products into the underlying intestinal tissue [14, 15]. This leads to activation of the resident macrophages, dendritic cells and T lymphocytes, release of pro-inflammatory cytokines and recruitment of circulating inflammatory cells. Local production and release of these cytokines, in turn, cause further disruption of the tight junction barrier by promoting endocytosis and degradation of claudin-1 and occludin proteins, the key tightening transcellular components of the tight junction apparatus [16, 19–21]. Thus, impairment of tight junction promotes inflammation and inflammation damages tight junction, creating a self-perpetuating vicious circuit in these conditions.

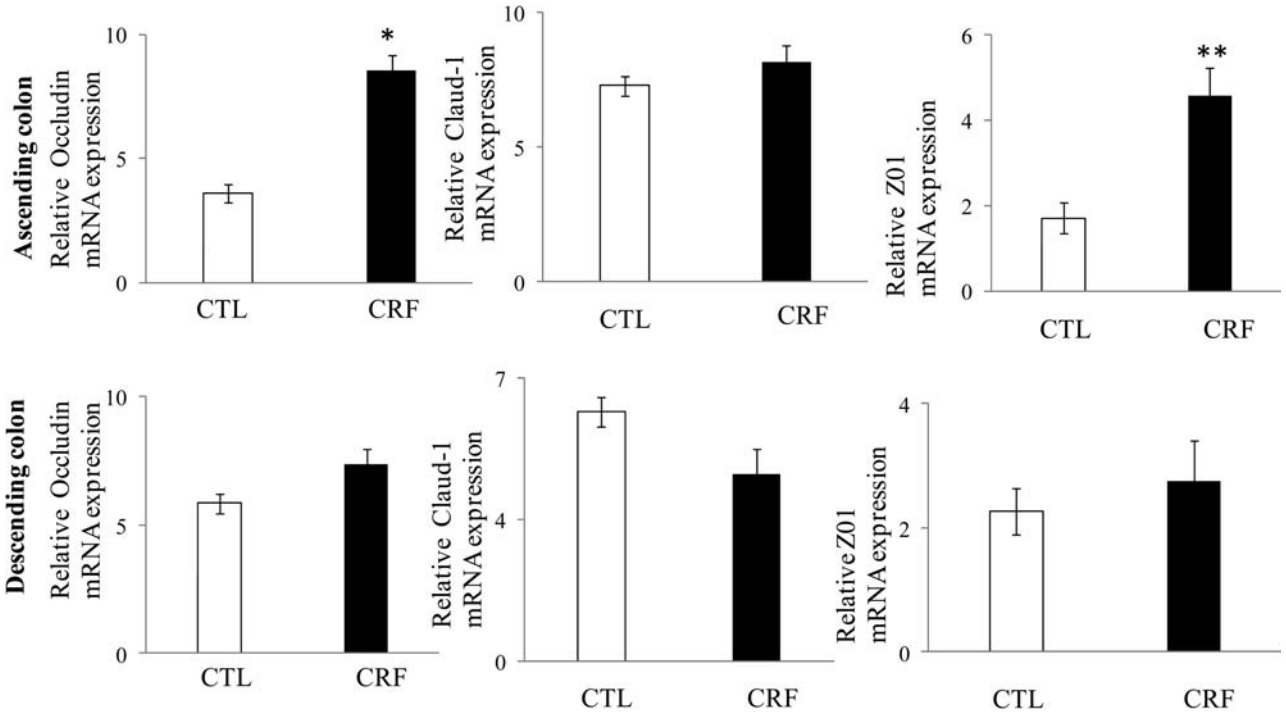

Fig. 5. Bar graphs mRNA abundance of occludin, claudin-1 and ZO-1 in the ascending and descending colon of rats with CKD induced by subtotal nephrectomy and sham-operated control rats. Each data point represent the mean ± SE of at least three separate experiments involving at least four sets of rats. *P < 0.05, **P < 0.01.

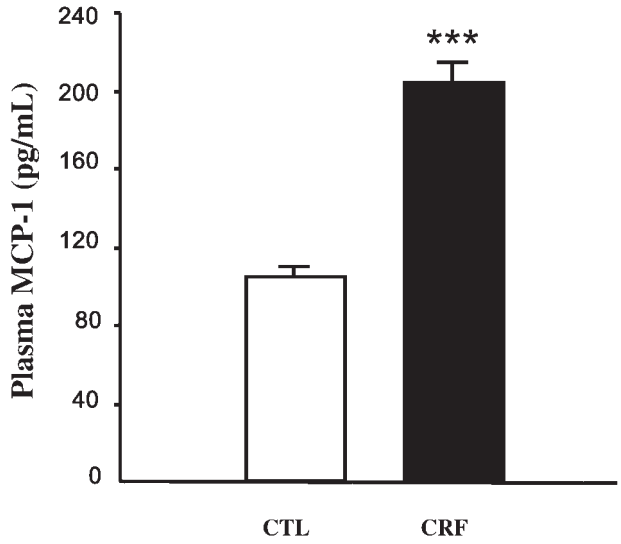

Fig. 6. Bar graphs depicting plasma MCP-1 concentration in the CKD and control (CTL) groups. *** P < 0.001, *N* = 6 animals in each groups.

As noted earlier, there is indirect but compelling evidence supporting the association of advanced CKD with increased intestinal permeability [6–12]. The marked reductions in protein abundance of claudin-1 and occludin, the key transcellular components, and of ZO-1, the main cytosolic plaque component of the tight junction, shown for the first time here, provides direct evidence for the disintegration of the intestinal tight junction in uremia.

Occludin is an integral component of the tight junctional complexes that regulates epithelial integrity and paracellular barrier function. Expression of occludin is markedly decreased in intestinal permeability disorders, including Crohn's disease, ulcerative colitis and celiac disease, suggesting that the decrease in occludin expression may play a role in the increase in intestinal permeability. The involvement of occludin in intestinal epithelial tight junction barrier is supported by experiments showing increased transepithelial flux rate of macromolecular probes with selective knock down of occludin in cultured enterocytes and *in vivo* recycling perfusion of mouse intestine using occludin small-interfering RNA transfection. These observations point to the critical role of occludin in the maintenance of the tight junction barrier through the large-channel pathway, which is responsible for the macromolecular flux [22]. The marked depletion of occludin in both models of CKD shown here can, therefore, account for the increase in intestinal wall permeability and leakage of luminal bacterial endotoxins and other noxious luminal products in advanced CKD.

Claudins belong to a large family of tetra-spanning membrane proteins that constitute a major structural component of the tight junction and play a critical role in the paracellular diffusion of small aqueous molecules. Claudins have four transmembrane domains and their N- and C-terminals are oriented toward the cytoplasm. Interactions of their C terminus with the ZO-1 and ZO-2 proteins are essential for their assembly into tight junction. While some members of the claudin family including claudins 1, 3, 4, 5 and 8 serve to tighten epithelial barrier and thereby limit the paracellular flux of small aqueous

molecules, others namely claudin-2, -7 and -12 enhance permeability by forming channels. Under normal condition, colon has the highest expression of 'tightening' claudins [23]. Expression of the leak forming claudins e. g. claudin-2 is markedly elevated, whereas that of the tightening claudin is reduced in inflammatory bowel diseases and certain other diarrheal states [14–16, 19, 20]. The colonic mucosa in our uremic animals showed a marked reduction in claudin-1, which is the main tightening member of the claudin family. Thus, downregulation of claudin-1 contributes to the impairment of tight junction permeability barrier in uremia.

As noted in the introduction section, ZO-1 is the principal component of the cytosolic plaque protein, which contains multiple protein interaction domains with which it binds to the transmembrane proteins and to the underlying peri-junctional actomyosin ring and as such is essential for the assembly and function of the tight junction. ZO-1 expression was significantly reduced in the colonic tissue of our uremic rats. Downregulation of ZO-1 must, therefore, contribute to impaired intestinal barrier function in advanced renal failure.

The observed depletion of the key protein constituents of tight junction elucidates the molecular mechanisms of the uremia-induced impairment of intestinal barrier function shown in the earlier studies [6–13] and its contribution to the systemic inflammation and common occurrence of endotoxemia in advanced CKD.

The reduction in the abundance of the measured tight junction proteins in the colonic epithelium of the uremic animals was accompanied by their normal or elevated mRNA abundance. This observation suggests that reduction in the abundance of the given proteins may be due to post-transcriptional or post-translational alterations of these molecules. It is of note that inflammatory cytokines such as interferon-gamma, tumor necrosis factor-alpha, interleukin (IL)-12 and IL-1beta can increase tight junction permeability via depletion of transcellular components of the intestinal tight junction by promoting their endocytosis and degradation and/or by upregulating myosin light chain kinase [19]. Given the prevailing systemic inflammation which is a constant feature of advance CKD, this phenomenon may, at least in part, contribute to the post-translational depletion of colonic claudin-1 and occludin in uremia. However, the observed reduction of phosphorylated myosin light chain in the colonic tissues of the CKD rats tends to argue against inflammation as the primary driver of uremia-induced depletion of the tight junction proteins. Further studies are planned to explore the mechanism(s) of uremia-induced depletion of the tight junction proteins.

Bowel wall edema and ischemia in patients with uncompensated congestive heart failure or hepatic cirrhosis have been shown to increase intestinal permeability and lead to endotoxemia, systemic inflammation and even bacterial translocation [24–26]. Fluid retention and hypoalbuminemia (caused by either proteinuria or inflammation/malnutrition syndrome) frequently result in edema formation in patients with CKD. Therefore when present, severe hypervolemia and edema can impair intestinal barrier function. It should be noted, however, that due to severe tubulo-interstitial injury, animals with adenine-induced CKD had marked urinary concentrating defect and polyuria. Similarly, urine output was significantly increased in animals with CKD induced by 5/6 nephrectomy. Consequently, hypervolemia and edema were absent and could not account for the observed intestinal tight junction defect in these models.

It is well known that endotoxin and endotoxin fragments contained in the dialyzate solutions can enter the systemic circulation across the high-flux dialyzer membranes during hemodialysis treatment [27]. In addition, iatrogenic episodes of intra-dialytic hypotension caused by vigorous ultrafiltration can lead to bowel ischemia and hence gut barrier dysfunction. If present, by facilitating leakage of endotoxin and other noxious luminal contents, disintegration of intestinal epithelial tight junction apparatus can contribute to the prevailing inflammation and its adverse consequences in the ESRD population.

In conclusion, uremia results in disintegration of the colonic tight junction apparatus, a phenomenon which can contribute to the systemic inflammation and account for the previously demonstrated evidence of defective intestinal barrier function in humans and animals with advanced CKD. Further studies are planned to explore the effect of uremia in other segments of the gastrointestinal tract.

Conflict of interest statement. None declared.

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Uraemia disrupts the vascular niche in a 3D co-culture system of human mesenchymal stem cells and endothelial cells

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Abstract

Background. Recent studies identified mesenchymal stem cells (MSC) as major players in vascular remodelling and the sub-endothelial compartment as the stem cell niche. The uraemic microenvironment predisposes to increased levels of reactive oxygen species, accelerated ageing of endothelial cells (EC) and vascular sclerosis.

Methods. We generated an *in vitro* model of a vascular niche consisting of a three-dimensional collagen I/III gel containing MSC and EC. We recapitulated a uraemic microenvironment by supplementing the medium with 20% pooled sera from either healthy or uraemic patients.

Results. Under healthy conditions, MSC/EC co-culture in collagen gels resulted in vessel-like tube formation. In contrast, uraemic serum-induced expression of extracellular matrix proteins (real-time reverse transcription–polymerase chain reaction, immunohistochemistry) that was accompanied by significant collagen contraction and a myofibroblastic phenotype of MSC. Although the uraemic culture conditions stimulated EC proliferation (cell counting, BrdU assay) and did not induce apoptosis (7-amino-actinomycin, annexin V FACS analysis), the tube formation was disrupted in spite of significantly enhanced vascular endothelial growth factor-A messenger RNA expression. The excessive matrix synthesis and remodelling by uraemia-exposed MSC/EC was reminiscent of remodelling processes observed in arteries of 12 dialysis patients (using arteries from 10 children and 10 age-matched non-dialysis patients as controls).

Conclusion. Our data indicate a potential role of the sub-endothelial niche and its major cell types EC and MSC in the remodelling process of the vascular wall in CKD.

Keywords: extracellular matrix remodelling; human mesenchymal stem cell; human umbilical cord vein endothelial cell; uraemia; vascular sclerosis