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The Role of Oxidative Stress and Inflammation in Acute Oxalate Nephropathy Associated With Ethylene Glycol Intoxication

To the Editor: Calcium oxalate crystal (CaOx) deposition within the renal parenchyma is well described as a cause of both acute kidney injury and chronic kidney disease. Animal and cell line studies have previously demonstrated that this injury is modulated by oxidative stress–induced danger-associated molecular patterns following CaOx deposition within the interstitium[1,2]. It has been postulated that these oxidative stress-induced danger-associated molecular patterns may participate in the pathogenesis of acute kidney injury and chronic kidney disease in humans, leading to the recruitment of an inflammatory response characterized by an increased infiltration of monocytes and macrophages.[1,3,4] However, evidence of this mechanism in humans is lacking. We report the morphological changes in renal cortical tissue from a 36-year-old woman with acute oxalate nephropathy secondary to ethylene glycol intoxication, and correlate them with a marker of oxidative stress and cell populations in an associated inflammatory infiltrate. Our findings provide initial evidence linking renal deposits of CaOx to oxidative stress and inflammation in humans.

A 36-year-old woman was brought to hospital after being found unresponsive at home. She was noted to have acute kidney injury (serum creatinine 789 μmol/l) and a severe metabolic acidosis (pH 7.09; HCO_3 2 mmol/l; pCO_2 5 mm Hg). She had no relevant past medical history and had a previously recorded serum creatinine of 51 μmol/l. As the cause of her acute kidney injury was unknown, a renal biopsy was performed on day 7 of her admission, which demonstrated acute oxalate nephropathy with significant deposition of CaOx in the renal parenchyma. Further investigation demonstrated a toxic level of serum ethylene glycol (8.8 mmol/l). She required 14 days of intermittent hemodialysis before renal recovery, and her serum creatinine remained elevated (102 μmol/l) 3 months after discharge.

To investigate the relationship between oxidative stress and the recruitment of inflammatory cells following acute oxalate nephropathy, excess fresh renal cortical tissue from the biopsy was obtained with informed consent and institutional ethics approval (Royal Brisbane and Women's Hospital Human Research Ethics Committee 2006/072).

Flow-cytometric analysis was performed after digesting the biopsy tissue with 1 mg/ml collagenase P (Roche, Mannheim, Germany) in the presence of 20 μg/ml DNase I (Roche) (250-μl volume) for 15 minutes and then further digesting with a mixture of 10 μg/ml trypsin and 4 μg/ml ethylenediamine tetraacetic acid (Life Technologies, Grand Island, NY) (500-μl volume) for 10 minutes (Figure 1). Single-cell suspensions were initially stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Life Technologies) to exclude nonviable cells. Cells were then incubated with Human TruStain FcX Blocking Solution (Biolegend, San Diego, CA) at room temperature for 5 to 10 minutes and then stained on ice for 30 minutes with mouse antihuman CD45, CD14, CD3 antibodies in cold fluorescence-activated cell-sorting buffer (0.5% bovine serum albumin [Sigma, St. Louis, MO] and 0.02% sodium azide [Sigma] in phosphate-buffered saline solution). Cell acquisition was performed on an LSR Fortessa (BD Biosciences, Sparks, MD) and data analyzed with FlowJo software (TreeStar, Ashland, OR).

Immunofluorescence staining was performed on frozen 7-μm tissue sections that were fixed with 25% ethanol:75% acetone at room temperature for 5 minutes, followed by a protein block with Background Sniper Blocking Reagent for 30 minutes (Biocare Medical, Concord, CA) (Figure 1). Sections were subsequently probed with anti-CD3 (rabbit polyclonal IgG; Agilent Technologies, Santa Clara, CA) and anti-CD68 (monoclonal mouse IgG3; Clone PG-M1; Agilent) at room temperature for 1 hour. Fluorescent detection was obtained by secondary incubation with AlexaFluor-488 antimouse IgG and AlexaFluor-647 antirabbit IgG (Life Technologies) at room temperature for 30 minutes. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies). Slides were coverslipped in fluorescence mounting medium (Agilent Technologies). A Zeiss 780 NLO confocal microscope (Carl Zeiss, Hamburg, Germany) was used for fluorescence microscopy. Image acquisition and analysis were performed using ZEN software (Carl Zeiss).

Immunohistochemical staining was also performed on frozen 7-μm tissue sections that were fixed with 25% ethanol:75% acetone at room temperature for 5 minutes (Figure 1). Endogenous peroxidase activity was blocked with 1% H_2O_2 for 10 minutes, followed by a protein block with 2% bovine serum albumin in Odyssey Blocking Buffer (Li-Cor, Lincoln, NE). Sections were probed with anti–4-hydroxynonenal (4-HNE)

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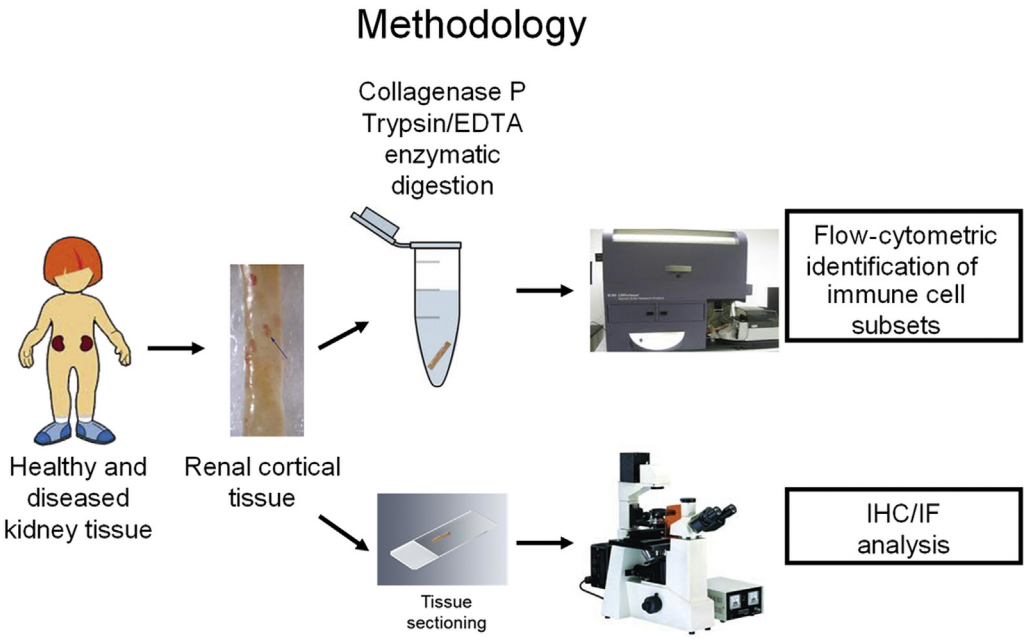

Figure 1. Study methodology. EDTA, ethylenediaminetetraacetic acid; IF, immunofluorescence; IHC, immunohistochemistry.

(goat polyclonal IgG; Abcam, Cambridge, MA) and anti−phosphorylated mixed lineage domain-like protein (p-MLKL) (monoclonal rabbit IgG; Abcam) at room temperature for 1 hour. Tissue sections were washed, and a goat or rabbit horseradish peroxidase polymer system (Biocare Medical) was applied according to the manufacturer's instructions. Peroxidase activity was developed with DAB substrate for 5 minutes. Sections were lightly counterstained with hematoxylin and mounted using DPX mounting medium. All analyses were compared to healthy renal cortical tissue as a control.

Polarized light microscopy confirmed the presence of CaOx deposits within the renal interstitium (Figure 2a) of our patient. There was a significant increase in 4-HNE renal expression compared to the healthy control (44.40 vs. 3.18 positive pixel intensity/μm^2; $P < 0.001$) with foci of higher 4-HNE renal expression co-localizing with the CaOx crystals (Figure 2b). Evidence from animal studies suggests that CaOx induces lipid peroxidation of the renal tubular membranes, which increases oxygen reactive species and consequently oxidative stress.[3,5,6] 4-HNE represents one of the most bioactive products of lipid peroxidation, and plays an important role mediating numerous signaling pathways including the inflammatory response.[7] The increased renal expression of 4-HNE in our patient is similar to that seen in these previous animal and cell line studies, and provides support for the same pathogenic mechanism occurring in humans.

In addition to inducing oxidative stress, CaOx crystal deposition has also been shown to cause direct cytotoxic effects on renal tubular cells, and this may lead to necrotic cell death.[8,9] The term "necroptosis" has recently been used to describe this regulated form of cell necrosis triggered by crystal deposition.[10] The signal transduction pathway of necroptotic cell death is mediated by ligation of tumor necrosis factor alpha to its receptor. This ultimately results in the phosphorylation of MLKL, which, once phosphorylated, translocates to the plasma membrane where it disrupts plasma membrane integrity.[8,10,11] The significant tubular positivity for p−MLKL found in our patient compared to the healthy control (Figure 2c) provides corroborating evidence for the role of necroptosis-mediated cell death from acute oxalate nephropathy. Furthermore, the strong tubular positivity of p−MLKL in our patient was associated with the increased expression of 4-HNE. This supports the recently emerging concept that necroptosis may trigger further inflammation, whereas reactive oxygen species may act as critical regulators of necroptotic signaling.[10,11]

Immunofluorescence demonstrated an increased infiltration of macrophages (CD68) and T cells (CD3) with foci of activity around calcium oxalate crystals (Figure 2d). There was a marked increase in leukocytes (26% vs. 3% $CD45^+$ cells) by flow cytometry in our patient compared to the healthy control (Figure 2e and f). These leukocytes were predominantly mononuclear cells (85% of leukocytes), of which 47% were T cells (CD3) and 29% were monocytes (CD14). Previous animal studies have shown that CaOx within the renal interstitium activates the immune system via

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