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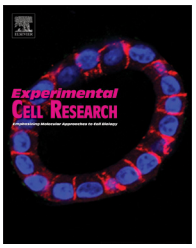

Research Article

Autophagy inhibition induces podocyte apoptosis by activating the pro-apoptotic pathway of endoplasmic reticulum stress

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ABSTRACT

Podocyte apoptosis is a major factor inducing podocyte depletion that predicts the progressive course of glomerulosclerosis. However, the molecular mechanisms underlying podocyte apoptosis are still not well understood. Autophagy is a lysosomal degradation system involving the degradation and recycling of obsolete, damaged, or harmful cytoplasmic materials and organelles. Recent advances in the understanding of the molecular processes contributing to autophagy have provided insight into the relationship between autophagy and apoptosis. However, their cross-talk remains largely obscure until now. Here, we found that podocytes both in vivo and in vitro always exhibited high basal levels of autophagy, whereas autophagy inhibition could induce podocyte apoptosis, suggesting the pro-survival role of autophagy in podocytes. Besides, we found that autophagy inhibition by 3-methyladenine (3-MA) could induce the activation of endoplasmic reticulum stress even without any external stimulations, whereas knockdown of CHOP could effectively improve podocyte apoptosis and down-regulated expression of slit-diaphragm proteins induced by autophagy inhibition. Collectively, this study demonstrated that autophagy might act as a crucial regulatory mechanism of apoptotic cell death by modulating the balance between the pro-survival pathway and the pro-apoptotic pathway of endoplasmic reticulum stress, which might provide a novel mechanistic insight into the interface between autophagy and apoptosis in the progression of podocyte injury.

Introduction

Podocyte is the most spectacular cell type whose location, architecture and relevance are unique [1]. In 1989, Fries et al. were the first to suggest that an inability of podocytes for cell replication in the adult represented the major factor underlying nephron degeneration [2]. In subsequent years, numerous studies have pointed to podocyte depletion as a hallmark of both primary and secondary forms of glomerulosclerosis [3]. Podocyte apoptosis, the early glomerular phenotype in the progression of diabetic and non-diabetic renal diseases, has now been suggested to contribute to progressive podocyte depletion and glomerulosclerosis [4]. However, until now, the molecular mechanisms underlying podocyte apoptosis in chronic progressive glomerular

Abbreviations: 3-MA, 3-methyladenine; STZ, streptozotocin; TBG, tail blood glucose; CHOP, CCAAT-enhancer-binding protein homologous protein; siRNA, small interfering RNA; ER, endoplasmic reticulum

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diseases remain largely unknown. Therefore, deeper understanding of the cell biology of podocyte will help us to develop rules on how to protect podocytes from apoptosis and halt the progression of chronic renal diseases.

Autophagy is a physiologically regulated and evolutionarily conserved process mostly implicated in the recycling of portions of cytosol and in the removal of superfluous or damaged organelles [5]. Because of its internal needs to maintain cellular homeostasis [6], autophagy is essential for the survival, differentiation and development. Dysregulated autophagy has been suggested to play pathogenic roles in a variety of diseases including cancer, neurodegeneration, diabetes, aging, heart disease and so on [7]. It is believed that autophagy is important in cell death decisions and could protect cells by preventing them from undergoing apoptosis [8,9]. Nevertheless, autophagy may also result in cell death called "autophagic cell death" through excessive self-digestion and degradation of essential cellular constituents under certain circumstances [10,11]. Thus, it seems that the relationship between autophagy and apoptosis is complex [12].

Our previous studies have demonstrated that high basal levels of autophagy were essential for podocyte biology and defective autophagy happening in the progression of diabetic nephropathy might facilitate podocyte injury [13]. In this study, we further demonstrated that defective autophagy was associated with podocyte apoptosis and the interplay between autophagy and apoptosis was important in the pathogenesis of diabetic podocyte injury. Since autophagy played an important role in endoplasmic reticulum quality control [14,15], here we indicated that defective autophagy commonly seen in diabetic nephropathy could induce the activation of endoplasmic reticulum stress. Knockdown of CHOP expression by siRNA not only prevented autophagy inhibition-induced podocyte apoptosis, but also restored autophagy inhibition-induced down-regulated expression of slit-diaphragm proteins. Our studies identified autophagy as a regulatory mechanism of apoptotic cell death by modulating the pro-survival pathway of endoplasmic reticulum stress. Autophagy inhibition in diabetes and aging might induce podocyte apoptosis by activating the pro-apoptotic pathway of endoplasmic reticulum stress.

Materials and methods

Animal model

As described previously [13], Male CD-1 mice weighed ~18–22 g were acquired from the Specific Pathogen-Free (SPF) Laboratory Animal Center of Nanjing Medical University. According to the guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health at Nanjing Medical University, animals were treated humanely and rendered diabetic by intraperitoneal injections of 50 mg/kg streptozotocin daily (STZ, Sigma, St. Louis, MO) for 5 days. For the sham-operated group, normal saline was administered. Tail blood glucose (TBG) levels were monitored consecutively and diabetic status was confirmed by the manifestation of weight loss, polyuria, and TBG level greater than 500 mg/dl. The diabetic mice were sacrificed at 12-weeks after the treatment; serum and urine were collected and the kidneys were harvested for various analyses.

Human renal biopsy specimens

Renal biopsy specimens were obtained from patients undergoing diagnostic evaluation at the Division of Nephrology, The Second Affiliated Hospital of Nanjing Medical University. A total of 6 subjects were (age range, 29–45) selected from our database with the criteria of having at least 10 glomeruli in the block available for histological sectioning. All biopsy specimens were evaluated by a single pathologist who was unaware of the results of molecular studies. The patients ($n=3$) with mild mesangium proliferative glomerulonephritis whose proteinuria were about 0.32 ± 0.174 g/24 h were chosen as a control group. The diabetic patients ($n=3$) whose proteinuria were about 5.21 ± 1.863 g/24 h were chosen as a diabetes group.

Cell culture and treatment

The conditionally immortalized mouse podocyte cell line was kindly provided by Dr. Zhihong Liu (Research Institute of Nephrology, Nanjing General Hospital of Nanjing Military Command, Nanjing, Jiangsu Province, China) while their cell lines were provided by Dr. Peter Mundel (Mount Sinai School of Medicine, New York, NY, USA) and described previously [16]. Cells were cultured at the permissive temperature (33 °C) in RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY) and recombinant interferon-γ (Invitrogen, Carlsbad, CA, USA). To induce differentiation, podocytes were grown under non-permissive conditions at 37 °C for 10–14 days in the absence of interferon-γ. After serum starvation for 16 h, cells were exposed to the treatment for indicated time periods.

Immunofluorescence staining

Indirect immunofluorescence staining was performed according to an established procedure [17]. For immunofluorescence staining of kidney sections, cryosections at 5 μm thickness were prepared and fixed in cold methanol/acetone (1:1) for 10 min. After being blocked with 2% normal donkey serum in PBS for 40 min, the sections were incubated with primary antibodies against LC3 (no. 2775S, Cell Signaling), laminin (L9393, Sigma-Aldrich), podocin (P0372, Sigma-Aldrich), WT1 (sc-192, Santa Cruz Biochemical) and cleaved caspase-3 (no. 9661S, Cell Signaling), respectively, in PBS containing 1% BSA overnight at 4 °C. As a negative control, the primary antibody was replaced with either nonimmune mouse or rabbit IgG, corresponding to species of the primary antibodies. Sections were then washed thoroughly in PBS and incubated with FITC and TRITC-conjugated secondary antibody (Sigma-Aldrich) at a dilution of 1:500 in PBS containing 1% BSA(bovine serum albumin) in the dark for 1 h. After being thoroughly washed with PBS, slides were mounted with VECTASHIELD® anti-fade mounting media (Vector Laboratories, Burlingame, CA) and viewed with a Nikon Eclipse 80i Epi-fluorescence microscope equipped with a digital camera (DS-Ri1, Nikon). In each experimental setting, images were captured with identical light exposure parameters and aperture settings.

Cells cultured on cover slips were washed twice with cold PBS and fixed with cold methanol/acetone (1:1) for 10 min at −20 °C. Following three extensive washings with PBS, the cells were blocked with 2% normal donkey serum in PBS buffer for 40 min at room temperature. Then the cells were incubated with

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