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Research article

Autophagy protects podocytes from sublytic complement induced injury

Qianying Lv [a], Fengjie Yang [a], Kun Chen [b], Yu Zhang [a,*]

[a] Department of Pediatrics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
[b] Department of Anesthesiology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

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ABSTRACT

Podocyte injury induced by sublytic complement attack is the main feature of membranous nephropathy (MN). This study aimed at investigating the impact of sublytic complement attack-related autophagy on podocyte injury in vitro. Here, we show that sublytic complement attack enhances MPC5 podocyte autophagy in vitro. Inhibition of autophagy by treatment with 3-methyladenine (3-MA) significantly increased sublytic complement attack-induced changes in the injury-related morphology, stress fiber, and podocyte apoptosis, but decreased the survival and adhesion of MPC5 podocytes. In contrast, promotion of autophagy by treatment with rapamycin mitigated sublytic complement attack-induced changes in the injury-related morphology, stress fiber, and podocyte apoptosis, but increased the survival and adhesion of MPC5 podocytes. These data suggest that autophagy may protect podocytes from sublytic complement attack-induced injury in vitro.

1. Introduction

Membranous nephropathy (MN) is one of the most common causes of idiopathic nephrotic syndrome. Although about 30% of the patients with MN may undergo spontaneous remission, most of them can progress into end-stage renal failure within 10 years. The pathogenesis of MN is characterized by the deposits of immunocomplex in the lamina rara externa of the glomerular basement membrane (GBM), leading to membrane-like thickness of capillary wall. During the process of MN, podocytes, the glomerular visceral epithelial cells, are damaged because immunocomplex can activate complement cascade by inserting sublytic quantities of C5b-9 into their membranes [1]. Prolonged podocyte injury can accelerate the pathogenesis of kidney diseases, such as glomerulosclerosis [2]. However, the pathological process of podocyte injury and physiological regulation has not been clarified.

Autophagy is a highly conserved lysosomal pathway involved in the recycling of cytosol and the removal of superfluous or damaged organelles. Autophagy regulates the survival, differentiation, development and homeostasis of many types of cells. Dysregulated autophagy contributes to the pathogenesis of many diseases, including cancer, neurodegeneration, heart disease and certain kidney diseases [3]. Autophagy is important to degrade large structures, and critical for the cellular refreshing [4], particularly for quiescent and terminally differentiated cells, such as neurons and glomerular epithelial cells [5]. A previous study has shown high levels of autophagic process in podocytes in animals with MN, passive Heymann nephritis [6]. Inhibition of autophagy deteriorates the pathogenesis of these diseases with severer clinical symptoms, such as proteinuria, extensive foot-process effacement, and loss of podocyte. Accordingly, we hypothesize that autophagy may protect podocytes from the progression of MN. In this study, we employed a cellular model to mimic MN in vitro to determine the role of autophagy in podocyte injury during the process of MN.

2. Materials and methods

2.1. Cell culture

Immortalized mouse podocyte cells (MPC5) were kindly provided by Dr. Peter Mundel (Massachusetts General Hospital, Boston, Massachusetts, USA) and were cultured, as previously described [7]. Briefly, podocytes were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin

* Correspondence to: Department of Pediatrics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, No.1095, Jiefang Ave., Wuhan 430030, P. R. China.
E-mail address: yuzhang497@163.com (Y. Zhang).

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and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, USA). To propagate, the cells were cultured at 33 °C and treated with 10 U/ml of mouse recombinant γ-interferon (IFNγ, Pepro Tech, England) to stimulate their proliferation. When they reached at 80–90% confluence, podocytes were harvested, washed and cultured in a plate coated with type I collagen at 37 °C for at least 12 days to allow their differentiation. The differentiated cells were used for the following experiments.

2.2. Induction of sublytic C5b-9 (sC5b-9) attack in podocytes

A cell model of MN was established by inducing sC5b-9 attack in podocytes. Podocytes (5×10^5 cells/well) were incubated at 37 °C for 1 h with 1:100 diluted rabbit anti-MPC5 sera, prepared as described previously [8] and exposed to different dilutions (1:20–1:400) of human sera from healthy subjects (a complement source) at 37 °C for 1 h. Heat-inactivated human sera served as negative controls. After being centrifuged, the supernatants of incubated podocytes were collected and the concentrations of lactate dehydrogenase (LDH) released by dead cells were determined by measuring the values of optical density (OD) at absorbance of 490 nm using a LDH assay kit, according to the manufacturers' instruction (Biyuantian Biology and Technology, China). The cytolysis rates in individual samples were calculated by the formula, Cytolysis (%)= (OD of experimental LDH release – Background OD values)/(Maximum OD values-Background OD values) × 100%. A cytolysis rate of ≤5% was defined as sublytic attack [9]. To induce or inhibit autophagy, podocytes were pre-treated with rapamycin (10 ng/ml) or 3-MA (5 mmol/L, Sigma-Aldrich) for 1 h and treated with rabbit anti-MPC5 sera. Accordingly, there were seven groups of cells, including sC5b-9, sC5b-9/rapamycin, sC5b-9/3-MA, negative control with heat-inactivated human sera, 3-MA alone, vehicle DMSO and untreated control.

2.3. Western blot

The levels of LC3-I, LC3-II and p62 in the different groups of cells were determined by Western blotting. Briefly, the cell lysates (30 μg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels and transferred onto polyvinylidene difluoride (PVDF) membranes. After being blocked with 5% fat-free dry milk, the membranes were incubated with rabbit anti-LC3, rabbit anti-p62 (Cell Signaling, Danvers, MA) and rabbit anti-GAPDH (Epitomics, USA). The bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, USA) and visualized using an enhanced chemiluminescent reagent. The relative levels of target to control GAPDH were analyzed by densitometric scanning using WCIF ImageJ software.

2.4. Cell morphology analysis

The different groups of podocytes were stained with Wright–Giemsa staining solution (Sigma-Aldrich) and examined under an optical microscope (Olympus, Japan). TEM was also performed, as described previously [10]. The different groups of podocytes were fixed sequentially with 3% glutaraldehyde, post-fixed in 1% OsO4, dehydrated in acetone, and embedded in Epon812. The ultra-thin (80 nm) cellular sections were stained with uranyl acetate/lead citrate and visualized using a Hitachi H-600IV electron microscope (Hitachi Instrument, Japan, × 10,000).

2.5. Fluorescent staining and confocal microscopy

The different groups of podocytes in 6-well plate were stained with 0.05 mmol/L monodansylcadaverine (MDC, Sigma-Aldrich) at 37 °C for 20 min, fixed in 4% paraformaldehyde for 10 min and examined for intracellular autophagic vacuole using a fluorescence microscope (Olympus, Japan).

To stain the cytoskeletal proteins of F-actin, the different groups of podocytes were fixed with pre-warmed 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 5% BSA. Subsequently, the cells were stained with rhodamine phalloidin (1:20, Cytoskeleton, USA) for 30 min at room temperature. The cells were evaluated using a confocal microscope (Olympus).

2.6. Cell viability assay

The viability of cells was measured by MTT assay. Approximately 2×10^3 cells were cultured in 96-well plates and treated in triplicate with various reagents (see above) for 48 h. During the last 4 h incubation, the cells were exposed to MTT (5 mg/ml, Sigma-Aldrich) and the resulting formazan in each well was dissolved in 200 μl DMSO, followed by measuring the absorbance at 570 nm using a microplate reader (BioTek, USA). The survival rates of different groups of cells were calculated by (OD values of the experimental samples/OD values of the control) × 100%.

2.7. In vitro adhesion assay

The impact of autophagy on adhesion of podocytes was determined by adhesion assay, as described previously [11]. Briefly, differentiated podocytes (2×10^3 cells/well) were cultured in triplicate in complete medium in 96-well plates that had been precoated with collagen I (10 μg/ml) or 1% bovine serum albumin (as control) at 37 °C for 1 h. After being washed with PBS to remove the unbound cells, the adhered cells were fixed in formalin. The numbers of adhesive cells in individual groups were examined by two independent researchers in a blinded manner. The data was expressed as the mean ± SD of each group (12 independent biological replicates) from three separate experiments.

2.8. Apoptosis assay

The percentages of apoptosis in the different groups of cells were determined using an FITC-Annexin V/PI Apoptosis Detection Kit, according to the manufacturers' instruction (Nanjing KeyGen Biotech, Nanjing, China). Briefly, the different groups of cells (5×10^5/tube) were stained in duplicate with FITC-Annexin V/PI for 15 min in the dark and the percentages of apoptotic podocytes were characterized by flow cytometry in a FACScan flow cytometer (BD Biosciences, USA) using Cell Quest TMPro software (BD Biosciences, USA).

2.9. Statistical analysis

Data are present as the means ± SD. All experiments were performed at least 3 times. The difference among the groups was analyzed ANOVA and posthoc Bonferroni test and the difference between two groups was determined by Student's *t*-test using SPSS version 13.0 statistical software. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Successful induction of sublytic complement attack in podocytes

The IgG antibodies against membrane surface antigens on podocytes in the subepithelial space form subepithelial immune complexes to trigger activation of the complement cascade and