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Fibrillogenic amylin evokes the apoptosis of human mesangial cells

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Abstract

Objective: To explore the apoptotic role of amylin on human mesangial cell (MC).
Materials and methods: Primarily cultured human MCs were applied and treated with fresh amylin preparation. Human MCs were identified by the morphology and immunofluorescence staining. The apoptotic cells were determined by ultrastructure changes, TUNEL, and DNA fragmentation analysis. Propidium iodide staining and flow cytometry was employed for quantitative measurement of apoptosis.
Results: Under the light and transmission electronic microscopy (TEM), the human MCs with condensed chromatin, plasma shrinkage, marginated nuclear chromatin or apoptotic body were observed in amylin-treated MCs. Positive TUNEL staining, hypolipoid DNA peak, and typical DNA ''ladder'' pattern were also detected in amylin-treated MCs. Quantitative analysis of the apoptotic MCs showed that human amylin induced an increase of the percentage of apoptotic cells in a dose-dependent manner. Amylin nano-scale fibrils (5–18 nm) in diameter were detected in the cultured solution using negative staining under the TEM. Compared to the control, no significant changes of lactate dehydrogenase release were observed in amylin-treated MCs ($P > 0.05$).
Conclusions: Fibrillogenic amylin evokes the apoptosis of human MCs *in vitro*, which may explain the mechanism of the hypocellular mesangial damage and progressive glomerulosclerosis of the patients with diabetic nephropathy.

Keywords: Amyloid; Apoptosis; Mesangial cell; Glomerulosclerosis; Diabetic nephropathy

1. Introduction

Type 2 diabetes mellitus (T2DM) is characterized by islet amyloid deposition and decreased β-cell mass. Such deposit can be detected in the pancreatic tissue of over 90% of patients with T2DM and its major constituent is amylin [1,2]. Amylin is a 37-amino acid polypeptide normally synthesized, packaged within secretory granules and co-secreted with insulin by the pancreatic islet β-cells in response to elevations in plasma glucose levels. Human amylin is highly amyloidogenic [2]. It has been demonstrated that amylin fibril deposits can induce the apoptosis of human islet β cells, which correlates with a loss of up to 50% of β-cell mass in the individuals with T2DM [1]. Thus, amylin may play an important role in the pathogenesis of T2DM.

Diabetic nephropathy (DN) is a common and devastating long-term complication of diabetes mellitus, which is currently the leading cause of end-stage renal failure requiring renal replacement and dialysis therapy [3]. Renal characteristic histological changes of

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DN include glomerular mesangial expansion with enhanced extracellular matrix deposition, mesangiolysis, Kimmelstiel–Wilson (K–W) nodules, and thicken glomerular basement membrane, which are closely associated with mesangial cell (MC) damage [4].

Insulin resistance, obesity and elevated serum levels of insulin and amylin are the clinical features of T2DM patients [1]. High prevalence of pancreatic islet amyloid was detected in end-stage renal failure patients with increased serum amylin level [5]. Adrenomedullin together with amylin is part of a peptide family characterized by some common biological activities and the presence of a six-amino acid ring structure formed by an intramolecular disulfide bond [6]. Recently, Plank et al. [7] reported that adrenomedullin could significantly reduce the number of MC through increasing the apoptosis. Our previous work demonstrated that amylin deposits in the area of glomerular mesangial expansion, K–W nodules, and glomerular sclerosis in the DN patients [8]. More importantly, those with renal amylin deposition had more severe glomerular nodular lesion and renal function deterioration [8]. These make it tempting to speculate that amylin fibril deposition could induce the apoptosis of mesangial cell. To confirm this, we examined the apoptotic effect of amylin on human MC *in vitro* using the technique of transmission electronic microscopy (TEM), TUNEL assay, DNA gel electrophoresis, and propidium iodide (PI) staining with flow cytometry.

2. Materials and methods

2.1. Primary mesangial cell culture

Glomeruli were isolated from normal human kidney deemed unsuitable for transplantation by differential sieving of renal cortical tissue. After collagenase treatment, glomerular segments were plated in culture media favoring the growth of MC as previously described [9]. MCs were identified by typical stellate morphology when subconfluent and typical hillocks at confluence as well as positivity for desmin and actin by immunofluorescence along with negative staining for cytokeratin and factor VIII (Synbiotics, USA). The human MCs with over 98% purity were maintained in RPMI 1640 (Gibco, USA) supplemented with 20% heated-inactivated fetal calf serum (Gibco, USA) and incubated at 37 °C in humidified 5% CO_2 in air.

2.2. Preparation of amylin solution

Synthetic full-length human amylin (H-7905, USA) was obtained from Bachem California Inc. and kept at −20 °C for less than 7 days before application to cells. The amylin peptide was dissolved in phosphate-buffered saline (pH 7.4) at the concentration of 1 g/L and then applied to cells within the 5 min of being dissolved [10]. All experiments were performed using the freshly dissolved amylin solution.

2.3. Morphological observation

For light microscopy assay, MCs samples were spun down on slide, air dried, fixed in 95% ethanol for 10 min, and then stained with hematoxylin and eosin. Apoptosis was identified by the presence of nuclear condensation. For TEM assay, MCs were fixed in 4% glutaraldehyde in 0.1% mol/L PBS (pH 7.2) for 3 h at room temperature, treated with 0.1% osmium tetraoxide for 1 h, and serially dehydraded with increasing concentrations of acetone, and then embedded in Epon815. Sections (70 nm) were picked up on acetone-cleaned 200 mesh copper grids, and stained with uranyl acetate and lead citrate. Apoptotic cells were identified by their morphological features. For assessment of amylin fibril, samples were prepared by the negative staining technique [11]. Briefly, amylin cultured supernatant was applied to on 400 mesh copper grids, blotted, negatively stained with 2% phosphotungstic acid in distilled water, washed, dried in air, and visualized under the TEM.

2.4. TUNEL assay

Apoptosis of MCs was detected by terminal deoxynucleotidyl transferase mediated dUTP biotin nick end labeling with flow cytometry [12]. After treatment with amylin (2.5 μmol/L) for 48 h, MCs were harvested, washed with PBS and then fixed in 1% paraformaldehyde for 15 min. The pellets were washed, resuspended in 0.5 mL PBS, added in 5 mL cold ethanol, and then kept frozen −20 °C for 24 h. The cells (2.5×10^4) were incubated with 0.1% saponin (Sigma) in 500 μL at room temperature (25 °C) for 15 min. After being washed with PBS, the pellets were incubated with 100 μL TDT (Pregma) reagent (100 nmol sodium dimethylarsine acid, 0.1 mmol DTT, 5 mmol chloride sodium, 0.05 g/L BSA, 1 mmol/L Biotin-dUTP, 20IU TdT nick transferase) at 37 °C for 1 h. The cells were washed, added in 100 μL labeling solution (0.6 mmol/L chloride sodium, 0.06 mmol/L sodium acetate, 0.1% Triton X-100, 5% non-fatted milk and 2.5 mg/L Avidin-FITC) and then incubated at 37 °C for 30 min. Following being washed with PBS (0.1% Triton X-100), the sample fluorescence was measured using the flow cytometry.

2.5. DNA cleavage detection assay

Apoptosis of MCs induced by amylin was identified by cleavage of oligonucleosomal chromatin, a biochemical hallmark of apoptosis. For DNA isolation, MCs (5.5×10^5) after incubation with amylin for 36 h were harvested and centrifuged at 200 × *g* for 5 min and then processed as previously described [13]. Before DNA precipitation, the aqueous phase