ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Early onset of podocytes apoptosis – a TEM study in streptozotocin-induced diabetic rats

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Abstract

Cell death types are usually defined by morphological criteria. Even though podocyte loss is associated with various cell death mechanisms, podocyte apoptosis is rarely detected. The purpose of this study is to evaluate whether morphological signs of apoptotic cell death could be detected in early streptozotocin-induced diabetes in rats kidneys. There were used five Wistar rats, and renal tissue samples were drawn after three weeks of disease and further evaluated in transmission electron microscopy (TEM). Podocytes damage was indicated by two major findings: foot processes effacement, viewed as loss of cell processes, and chromatin condensation and margination (partial karyo-pyknosis: peculiar nuclear morphologies – partly normal, euchromatic, and partly positive for karyopyknosis and nuclear shrinkage). Mitotic glomerular endotheliocytes were also encountered. Podocytes cell death commitment and detachment appeared as concomitant events. However, karyopyknosis is not a specific feature of apoptosis. Thus, further biochemical evaluations are needed to distinguish between different pathways of podocytes death.

Keywords: kidney, diabetic nephropathy, foot processes, renal glomerulus, podocytes detachment, TEM.

Introduction

Cell death types are defined by morphological criteria [1]. Apoptotic cell death is characterized by rounding-up of the cell, retraction of pseudopodes, chromatin condensation, karyorrhexis, and blebbing of plasmalemma that remains undamaged until later stages of apoptosis [1, 2]. In human diabetic samples, apoptosis involves epithelial cells of the proximal and distal tubules, endothelial cells, and interstitial cells [3]; however, as Mulay *et al.* (2013) commented, podocyte apoptosis is often suspected but it is rarely detected [4]. Podocyte loss is a key event in glomerular disorders [5]. Diabetic podocytopathy is characterized by proteinuria and albuminuria, glomerular hypertrophy and hyperfiltration, thickening of the glomerular basement membrane (GBM) with altered matrix composition, the reduction of the nephrin protein in the slit diaphragm, and effacement of foot processes of podocytes [6–9]. Eid *et al.* proved that exposure of mouse podocytes to high glucose levels causes apoptosis, about one third of the cells becoming apoptotic by 72 hours through the generation of reactive oxygen species via sequential upregulation of CYP4A and the NADPH oxidases Nox1 and Nox4 [10].

Tissue degeneration in diabetes mellitus begins in early stages; however, there is limited evidence on cell injuries in these stages [11]. It was however identified by immunohistochemistry, in streptozotocin-induced diabetes, a caspase-dependent mechanism of the renal tubular apoptosis [11].

As it was hypothesized that in diabetic nephropathy podocyte apoptosis is a key event, we aimed to better characterize morphological signs of apoptotic cell death (including podocyte apoptosis) in kidneys from rats with early streptozotocin-induced diabetes by a transmission electron microscopy (TEM) study.

Materials and Methods

Five adult Wistar rats weighting 350–400 g were used. The animals were adequately kept and fed. The streptozotocin (STZ) powder (Sigma-Aldrich Gmbh, Munich, Germany) was diluted in distilled water 1 mL/ 20 mg. The animals were injected with streptozotocin 55 mg/kg of the body weight intraperitoneally under general anesthesia with diethyl ether inhalation. Body weight and blood glucose concentration were measured before injection, three days, and weekly thereafter. For measurement of the blood glucose concentration, we have used Accu-Chek Active (blood glucose monitoring system by Roche Diagnostics GmbH, Mannheim, Germany) and Accu-Chek Active blood glucose test strips. Blood samples were taken from the tail vein. The body weight and blood glucose were measured weekly. After pre-anesthesia with ether, the animals were euthanized after three weeks by intracardiac injection of a veterinary euthanasia drug (0.2 mL of T-61, Intervet, Kirkland, Quebec, Canada). All procedures were approved by the Institutional Bioethics Committee.

Kidney samples were prepared for TEM, as previously

described [12]. The Formvar coated grids were examined in a Philips electron microscope EM 208S (acceleration voltage of 80 kV) and snapshots were taken using a video camera Veleta and the iTEM Olympus Soft Imaging System.

Results

On ultrathin cuts, we adequately identified the main components of the filtration barrier: endothelial cells (ECs), glomerular basement membrane (GBM), and podocytes (P). Foot processes of podocytes were applied on the GMB that was thickened, but uniform. We identified two morphologically distinctive types of podocytes, "dark" and "light" (Figure 1). The "light" podocytes were larger and peculiarly rich in Golgi complexes (Figure 1C); they had extremely rare foot processes emerging directly from the cell body, and applied on the GBM. These "light" podocytes were thus considered as positive for foot processes effacement. Both types of podocytes that were found had deeply invaginated nuclei but those in "light" podocytes were euchromatic, while those in "dark" podocytes were rather heterochromatic with condensation of chromatin (Figure 1). Loss of cell processes with rounding of cells and chromatin condensation, belong to the morphological pattern of apoptosis, suggesting that the two types of podocytes were in different stages of apoptotic processes. This staged pattern of podocytes morphologies was reinforced by intermediate morphologies of podocytes, with most foot processes effaced, and presented peculiar nuclear morphologies – partly normal, euchromatic, and partly positive for karyopyknosis and shrinkage (Figure 2). We also found swollen podocytes with large cytoplasmic vacuoles and effaced foot processes. Slit diaphragms, seemingly unaltered, were double or single (Figure 3). Swollen mitochondria, whorls, and lipid inclusions were occasionally found within podocytes. On the opposite side of the GBM, mitotic ECs were found, as demonstrated by the evidence of centrioles (Figure 4).

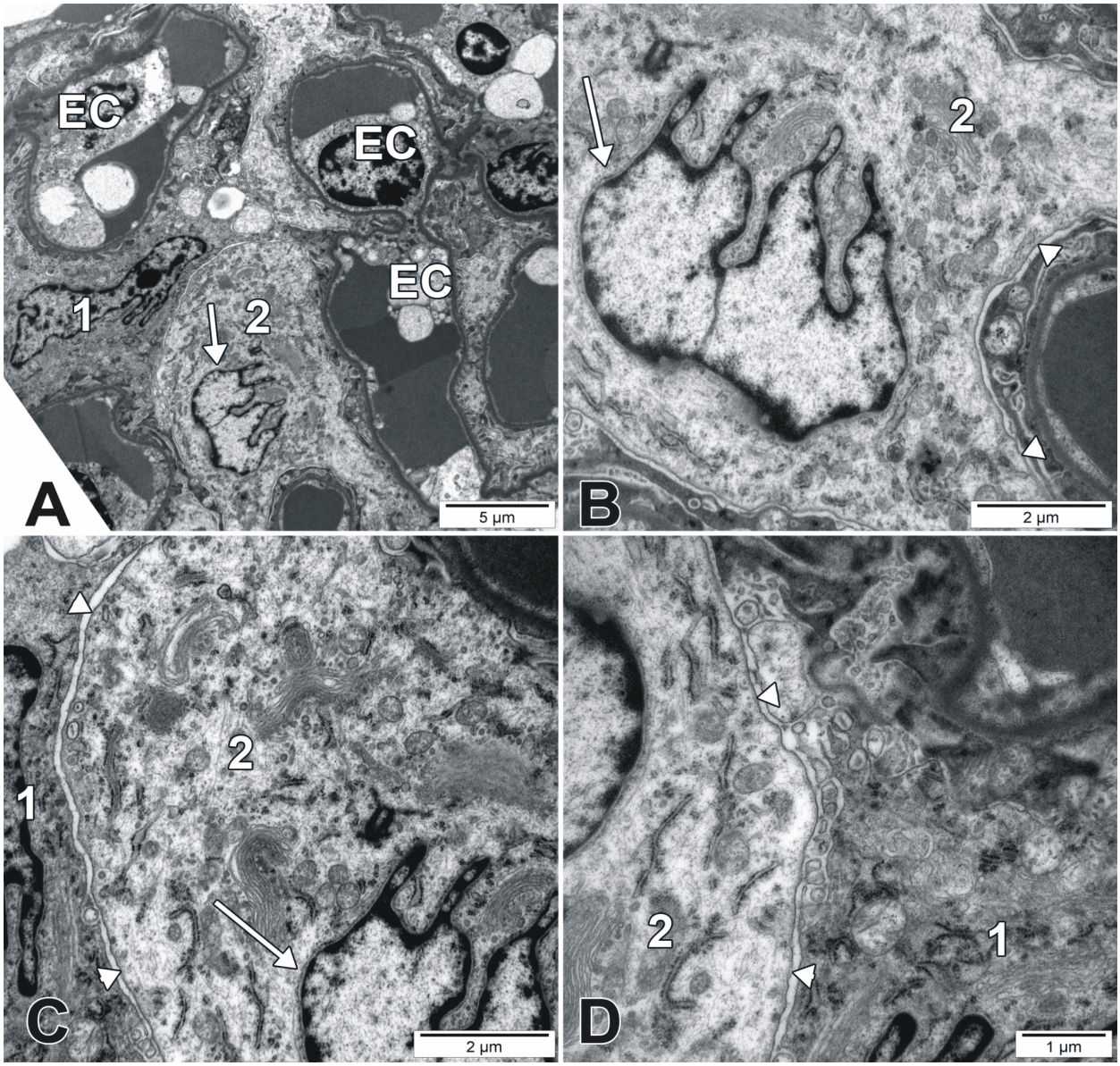

Figure 1 – ***Ultrathin cut of diabetic rat kidney. Within a renal glomerulus, "dark" (1) and "light" (2) podocytes are found (A), both presenting deep nuclear invaginations; however, the nucleus of the "dark" podocyte is also positive for karyopyknosis, while the nucleus of the "light" podocyte (arrow, A–D) is euchromatic. Almost complete foot processes effacement (arrowheads, B–D) was found in the "light" podocyte. EC: Endothelial cell.***

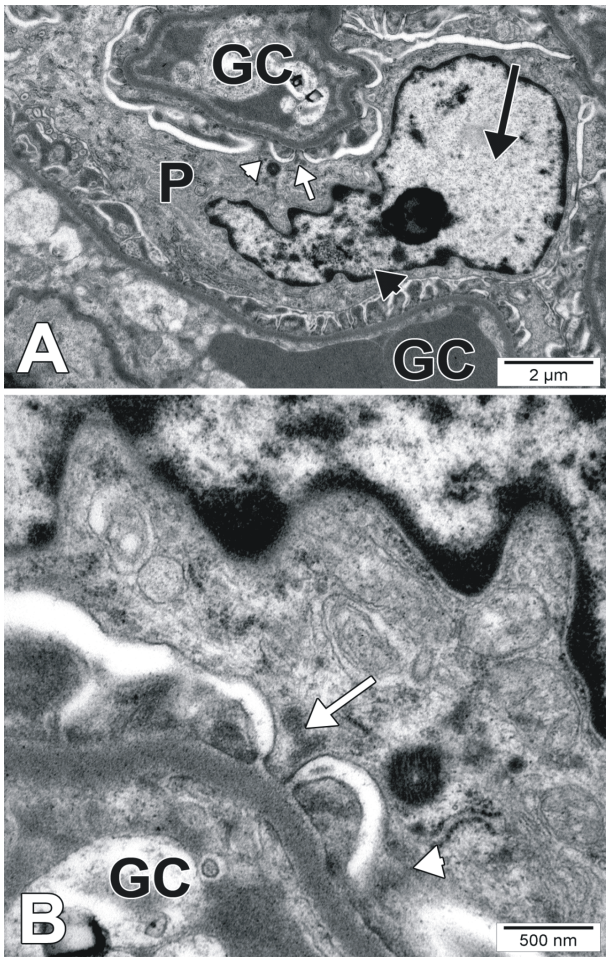

Figure 2 – *Ultrathin cut of diabetic rat kidney. (A) A podocyte (P) is identified between glomerular capillaries (GC). The nucleus of the podocyte is partly euchromatic (black arrow) and partly apoptotic (black arrowhead), with karyopyknosis and blebbing. The podocyte displays few foot processes (white arrow and arrowhead in A, detailed at higher magnification in B).*

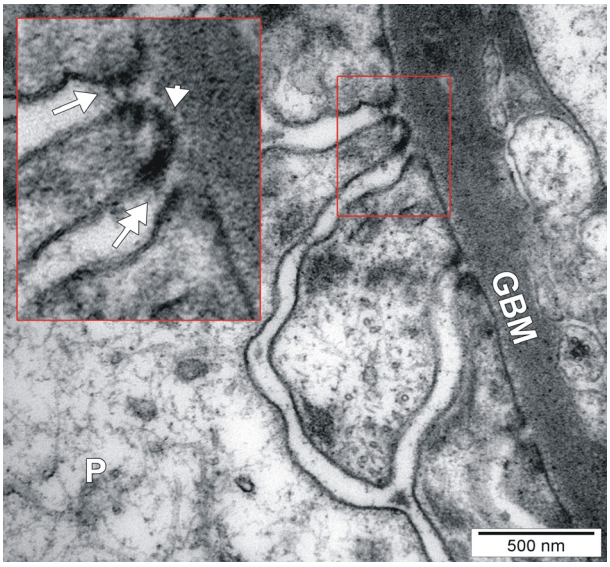

Figure 3 – *Ultrathin cut of diabetic rat kidney. A podocyte (P) foot process projects on the glomerular basement membrane (GBM). Cell–matrix interactions (arrowhead) are indicated. On opposite sides of the foot process double- (arrow) and single- (double-headed arrow) slit diaphragms are identified.*

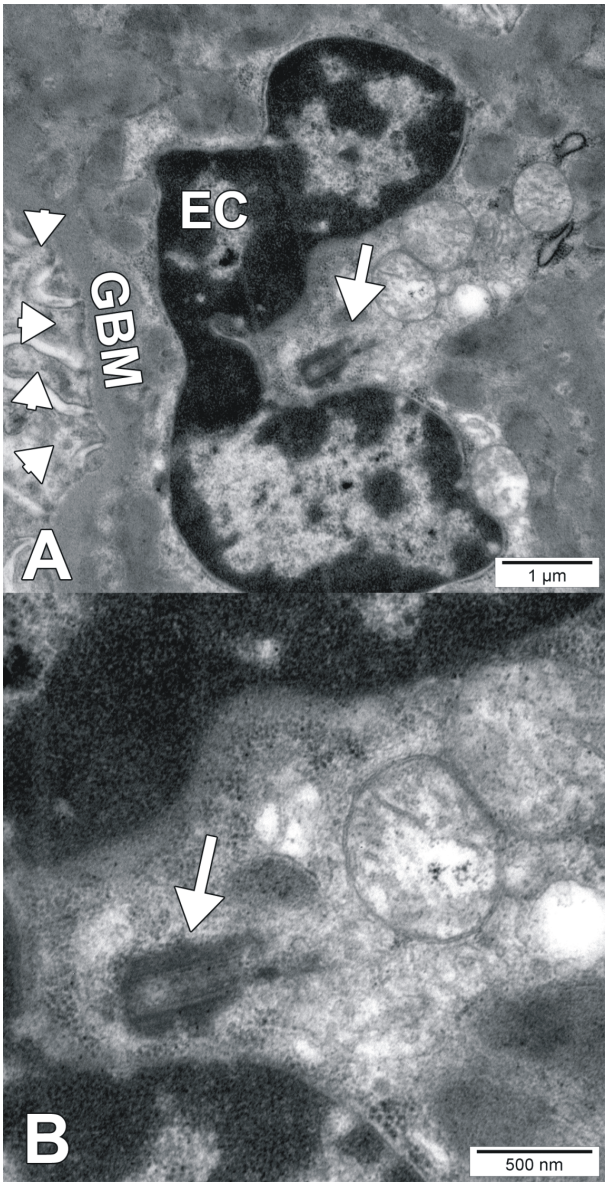

Figure 4 – *Ultrathin cut of diabetic rat kidney. (A) On each side of the glomerular basement membrane (GBM) foot processes of podocytes (arrowheads) and an endothelial cell (EC) are identified. The EC mitosis is suggested by the EC centriole (arrow). (B) The centriole (arrow) is detailed at higher magnification.*

Discussion

In a previous TEM study on diabetic mice (18 months of open hyperglycemia) were not identified apoptotic features of podocytes, being presumed that they were eliminated in the urine [9]. The authors speculated a staged morphological evolution of podocytes, beginning with podocytes hypertrophy and swelling [9]. The peculiar rich content of podocytes in Golgi complexes was also found in that experiment, suggesting the pattern to be unrelated to the duration of the disease. Swollen podocytes were thought to occur as a later aspect of cell injury [9]. These morphological aspects, as well as the lipid inclusions and effacement of foot processes were also found in the present study. However, in that study [9] the nuclear morphology of podocytes was overlooked. Attention was paid to the damaged processes of podocytes,

in order to support a hypothesis of podocyte atrophy [9]. If in TEM a podocyte process is not identified on the GBM, a cell process located within the urinary space and not applied on the GBM cannot be firmly diagnosed as being a podocyte process.

The density of podocytes is reduced in diabetic nephropathy, and podocyte apoptosis and detachment play pivotal roles in this event [13]. However, available data are scarce and a time sequence of events is unclear; viable podocytes detachment as well as podocytes detachment because of their apoptosis being concurrent theories [13]. We found here early signs of podocyte apoptosis in cells with effaced foot processes; these disappear later on during the apoptotic process. Podocytes apoptosis and detachment appeared us as concomitant processes, and may not succeed in a temporal sequence.

Viable podocytes were assessed in TEM in the urine of streptozotocin-induced diabetic rats, and further grown on culture media [14]. If foot processes effacement is considered as an apoptotic event, according to our theory, the differences between *in vivo* and *in vitro* conditions should be accounted for podocytes evolution, toward death or survival.

Podocyte apoptosis in the experimental diabetic glomerulopathy becomes detectable after four months of disease and is preceded by glomerular hypertrophy that induces compensatory podocyte hypertrophy, and associates podocytopathy and proteinuria. Podocyte apoptosis finally leads to podocytopenia and drives progression to expansion of the mesangium and glomerulosclerosis [15]. Mesangial and endothelial cells loss are known phenomena associated with diabetic nephropathy [15]. Our TEM results raise discussions on this pathogenic sequence, as signs of ongoing podocyte apoptosis and foot processes effacement were assessed at three weeks of disease duration. Jung *et al.* suggested recently that apoptosis occurs differentially in diabetic nephropathy, being faster in hypertrophic glomeruli [16].

Podocytes lacking insulin receptors are known to have increased apoptosis [17]. Insulin regulates the expression of vascular endothelial growth factor A (VEGF-A) in podocytes *via* the insulin receptor [18]. Podocytes are the main source of VEGF in renal tissue, with paracrine and autocrine activity on endotheliocytes and podocytes [17, 19]. Mesangial cells also produce VEGF and express VEGF receptors [8, 20]. Lack of VEGF-A in podocytes leads to loss of all major cell types in the glomerulus: endotheliocytes, mesangial cells and podocytes [17, 21, 22], but not exclusively. It is known that VEGF stimulates vascular endothelial cell proliferation [23]. In this regard, evidence of proliferating endotheliocytes in our samples may denote that even though there is podocyte loss at three weeks of diseases, the remaining podocytes continue to exert their influence on endotheliocytes. A possible role for adiponectin can be speculated here, as time as it was shown that it suppresses the VEGF-stimulated endothelial cell migration [24] and glomerular endothelial cells were found positive for constitutive adiponectin [25].

Pyknosis and karyorrhexis are common features of both apoptosis and oncosis [26]. Karyorrhexis is not pathognomonic for apoptosis [27]. Ischemic cell death is characterized by swelling; oncosis is cell death with swelling that is usually accompanied by karyolysis [27]. The morphological appearance of necrosis is frequently that of oncosis; oncosis (cell swelling), together with swelling of organelles and plasmallema rupture are main morphological traits of necrosis [1, 28]. *The Nomenclature Committee on Cell Death* (NCCD) limits the use of the term "oncosis" as it overlaps with necrosis, and with a partial apoptosis evolving into necrosis, and indicates that the term "necrosis" should be kept for "historical reasons". The NCCD recommends not to use the term of "apoptonecrosis" [1]. In these regards, we kept the term of "podocytes apoptosis", even though a degree of oncosis in podocytes with effaced foot processes was estimated.

This study partly reached its goal, identifying podocytes with early apoptotic changes, with foot processes effacement and karyopyknosis. However, there are limitations of the study, as in TEM it is quite difficult to perform a quantitative analysis and thus the observed podocytes alterations cannot be firmly related to diabetes. Further molecular studies should clarify whether or not different cell death modalities act in eliminating podocytes. The relative contribution of programmed versus mitotic cell death to podocyte loss is unknown [4]. Murine double minute-2 (MDM2)-dependent mitotic catastrophe was found mediating podocyte loss in adriamycin nephropathy [4].

Conclusions

Further ultrastructural studies should combine immuno-histochemistry to firmly relate the partly apoptotic phenotype of podocytes we found here with the molecular machinery of apoptosis.

Acknowledgments

This study was supported by the Sectoral Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/107/1.5/82839 (author #1).

All authors have contributed equally to this study.

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Received: October 16, 2013

Accepted: January 26, 2014