Original Paper

Response of Renal Podocytes to Excessive Hydrostatic Pressure: a Pathophysiologic Cascade in a Malignant Hypertension Model

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Key Words

Angiotensin-II • Apoptosis • TGF β_1 • Detachment/loss of viable podocytes • Podocin • Integrin β_1

Abstract

Background/Aims: Renal injuries induced by increased intra-glomerular pressure coincide with podocyte detachment from the glomerular basement membrane (GBM). In previous studies, it was demonstrated that mesangial cells have a crucial role in the pathogenesis of malignant hypertension. However, the exact pathophysiological cascade responsible for podocyte detachment and its relationship with mesangial cells has not been fully elucidated yet and this was the aim of the current study. **Methods:** Rat renal mesangial or podocytes were exposed to high hydrostatic pressure in an in-vitro model of malignant hypertension. The resulted effects on podocyte detachment, apoptosis and expression of podocin and integrin β_1 in addition to Angiotensin-II and TGF- β_1 generation were evaluated. To simulate the paracrine effect podocytes were placed in mesangial cell media pre-exposed to pressure, or in media enriched with Angiotensin-II, TGF- β_1 or receptor blockers. **Results:** High pressure resulted in increased Angiotensin-II levels in mesangial and podocyte cells. Angiotensin-II via the AT1 receptors reduced podocin expression and integrin β_1 , culminating in detachment of both viable and apoptotic podocytes. Mesangial cells exposed to pressure had a greater increase in Angiotensin-II than pressure-exposed podocytes. The massively increased concentration of Angiotensin-II by mesangial cells, together with increased TGF- β_1 production, resulted in increased apoptosis and detachment of non-viable apoptotic podocytes. Unlike the direct effect of pressure on podocytes, the mesangial mediated effects were not related to changes in adhesion proteins expression. **Conclusions:** Hypertension induces podocyte detachment by autocrine and paracrine effects. In a direct response to pressure, podocytes increase Angiotensin-II levels. This leads, via AT1 receptors, to structural changes in adhesion proteins, culminating in viable podocyte detachment. Paracrine effects of hypertension, mediated

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by mesangial cells, lead to higher levels of both Angiotensin-II and TGF- β 1, culminating in apoptosis and detachment of non-viable podocytes.

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Introduction

Previous studies have demonstrated that in the context of malignant hypertension, mesangial cells play crucial roles in kidney renal injury by overproduction of Angiotensin-II, consequently increasing apoptosis of adjacent cell populations [1]. Unlike mesangial cells, podocytes do not respond to high pressure by increased apoptosis [1]. However, both mesangial cells and podocytes respond to an increase in Angiotensin-II levels, which would seem to be sufficient for initiating apoptosis of both mesangial cells and podocytes [1]. During hyperfiltration and disease podocytes either adapt or are injured [2]. With respect to hypertension and high intra-glomerular pressure, podocytes play important roles in proteinuria and hypertensive nephrosclerosis [3].

The mechanism of podocyte injury remains unclear in context of hypertension and further research is needed to investigate the pathophysiologic cascade responsible for podocyte injury in the context malignant hypertension.

Podocytes reside in the glomerular basement membrane (GBM)[2] and constitute the final filtration barrier in the glomerulus. Their dysfunction has a pivotal role in various renal injuries. Together with mesangial cells, podocytes are the main resident cells of the glomerular tuft, and their loss signifies progression of acute renal damage towards end-stage renal failure [4, 5]. Following renal insult, mesangial cells retain the ability to proliferate and can partially recuperate [6]. By contrast, mature podocytes normally do not regenerate *in vivo* and therefore, any podocyte loss can culminate in long standing glomerular disease [4-7].

In response to injury, podocytes adaptively change and can undergo hypertrophy, dedifferentiation, detachment and apoptosis, dependent on the intensity and duration of the injury [8-10]. Detachment of podocytes from the GBM is the major mechanism responsible for renal diseases [5, 11]. Loss of viable podocytes may result from mechanical pressure [12], such as shear stress exerted on the foot processes by filtrate flowing through the filtration slits [12, 13]. The balance between adhesion and detachment of podocytes is regulated via Angiotensin-II dependent mechanisms [14, 15]. This can explain the massive detachment of viable podocytes seen in a majority of renal diseases induced by hyperfiltration, renal hypertrophy and glomerular hypertension [16].

Malignant hypertension, one of the most malicious forms of hypertension, is manifested by an acute elevation of systemic blood pressure, strongly associates with poor prognosis, and frequently culminates in deleterious renal injury [17, 18]. In “standard” systemic hypertension, the renin-angiotensin system (RAS) initially increases arterial blood pressure, but it is eventually suppressed via a feedback inhibition mechanism. This feedback mechanism is impaired in malignant hypertension due to an innate auto-regulatory control loss [19, 20], the consequence being the absence of an alternative for counteracting the deleterious consequences of RAS activation [21]. Angiotensin receptor 1 (AT1) is responsible for most of the pathophysiological actions of angiotensin II in the malignant hypertension scenario [22, 23]. High levels of RAS activation induce apoptosis of both mesangial and podocyte cells, causing major damage of adjacent non-contractile epithelial and endothelial renal cell populations [1, 24-26]. In addition, RAS and TGF- β are a potent fibrogenic cytokines that are upregulated in kidney diseases. Expression of exogenous TGF- β causes renal fibrosis [27, 28]. This is a universal finding in every type of chronic kidney disease (CKD), both in animal models and in humans. *In vitro*, TGF- β and Smad7 can stimulate apoptosis in podocyte cells [29]. In addition, Angiotensin-II induced extracellular matrix (ECM) accumulation is mediated by TGF- β which can also bind and activate AT1 and AT2 receptors [30].

The *in-vitro* model of malignant hypertension has allowed the investigation of paracrine

crosstalk among various renal cell populations and their role in RAS activation in pressure-induced renal injury [1, 24, 25]. The first studies were performed on podocytes and mesangial cells firmly attached to culture vessels. To the best of our knowledge, similar studies on detached viable podocytes due to high pressure insults have not been conducted, and the pathophysiologic cascade responsible for that detachment has not yet been fully elucidated.

The aim of the current study was to investigate the pathophysiologic cascade responsible for podocyte detachment and loss during malignant hypertension.

Materials and Methods

Cell cultures

Undifferentiated renal embryonic cells were isolated from the metanephroi of rat fetuses excised at day 12 of pregnancy, as previously described [1]. The cells were repeatedly washed in phosphate-buffered saline (PBS) not containing Ca or Mg salts, and maintained for 48h in Knock-Out™ DMEM selective medium (GIBCO Life Technologies, Bethesda, USA), specific for embryonic cell culture. Following primary outgrowth, the cells were dispensed in equal amounts into 25ml cell culture flasks. Following 48h, the cells were randomly divided into three groups, to be exposed to different types of culture media:⁷⁻⁹

1. Cells remaining in their original medium, Knock-Out™ DMEM supplemented with 4.5g/L D-Glucose, 0.1mM sodium pyruvate, 10% FCS, which is certified by the manufacturer as specific for maintenance of undifferentiated embryonic cells.
2. Cells grown in DMEM, highly specific for selective propagation of renal podocytes in culture [31], supplemented with 4.5g/L D-Glucose, 0.1mM sodium pyruvate, 10% FCS, non-essential amino-acids and 5mM sodium selenite.
3. Cells cultured in RPMI-1640 medium specific for selective culture of renal mesangial cells, supplemented with 20% FCS and L-Valine replaced by D-Valine

The cells were then treated with fresh portions of the same selective media every three days. Progression of cell differentiation towards the desired phenotype was supervised using fluorescent immunohistochemistry, flow cytometry and/or RT-PCR methods.

Pressure-loading apparatus

The pressure-loading apparatus consisted of a water bath supplied with a heating device and a pressure chamber placed into water preheated to 37°C. Constant air pressure within the chamber (160±3mmHg, chosen following a series of preliminary dose-response experiments) was maintained by two valves providing pressure control at the exit port of the gas container. The one-way valves of the exit port allowed constant air flow rate of 0.5 l/min. The air flow consisted of 20% O₂ and 5% CO₂. The temperature inside the pressure chamber was maintained at 37±0.28C by a thermostat placed within the water bath [24].

Application of high hydrostatic pressure

Some cell culture flasks containing either mesangial cells or podocytes were placed in a pressure-loading apparatus [24] for 1h, to mimic the incidence of malignant hypertension. The other flasks remained unexposed to pressure, to be used as controls.

Experimental design

Podocyte detachment following application of high hydrostatic pressure. Flasks containing cultured podocytes were subjected as follows:

1. Cells not subjected to high hydrostatic pressure (control);
 2. Cells exposed to high hydrostatic pressure for 1h;
 3. Cells not subjected to high hydrostatic pressure, but instead supplemented with 10⁻⁸M TGFβ₁;
 4. Cells not subjected to high hydrostatic pressure, but instead supplemented with 10⁻⁸M Angiotensin-II.
- 5-7. Cells exposed to one of the following receptor blockers 2h prior to the application of high hydrostatic pressure:

- Losartan, an AT-1 receptor blocker (10^{-6} M);
- PD123319, an AT-2 receptor blocker (10^{-6} M);
- A TGF β 1 receptor blocker (R&D Systems)

8. Podocyte cells not subjected to high hydrostatic pressure were exposed to medium from mesangial cells that were subjected to high hydrostatic pressure for 1h.

9-11. Cells exposed to one of the following receptor blockers 2h prior to the exposure to medium from mesangial cells that were subjected to high hydrostatic pressure:

- Losartan, an AT-1 receptor blocker (10^{-6} M);
- PD123319, an AT-2 receptor blocker (10^{-6} M);
- A TGF β 1 receptor blocker (R&D Systems)

Following 1h-pressure application, the cells were incubated at standard conditions for 24h. When the incubation was terminated, the cultures were handled as follows:

a) The cell culture media samples containing cells spontaneously detached from the flask bottoms during 24h incubation were separately collected and centrifuged at 2000 rpm for 10 minutes. Podocyte pellets detached from the flask bottoms during the 24h-incubation were differentially counted, viable vs. dead cells, using a 0.1% trypan blue exclusion protocol. Ten randomly chosen microscopic fields were used for calculating the mean differential cell count for each sample. Podocyte cell supernatants were aliquoted and stored at -80°C , to be used in further experiments.

Mesangial cell supernatants were aliquoted and stored at -80°C , to be used in further experiments and their cell pellets were discarded.

b) Studies on transmembrane adhesion proteins regulating podocyte detachment

We investigated changes in integrin β 1 and podocin expression, important structural proteins of the filtration slits within the glomerular filtration barrier. Podocyte monolayers were subjected to 2.5% trypsin digestion, neutralized by FCS, pelleted by centrifugation at 1000g at room temperature for 5min, and washed twice in ice-cold PBS. The pellets obtained were incubated with target antibodies specific for podocin (Protein Tech, Chicago, USA) or FITC-conjugated integrin β 1 (eBioscience, San Diego, USA) and propidium iodide (PI) to evaluate apoptosis. Flow cytometry was performed on a Beckman Coulter FC-500 (Krefeld, Germany), with data analysis by FlowJo software, an integral part of the coulter.

Assessment of TGF β ₁ and Angiotensin-II

TGF β ₁ and Angiotensin-II synthesis were measured in podocyte and mesangial cells supernatants by specific ELISA assays (Human/Mouse/Rat Angiotensin-II RayBio, Rat TGF β 1 ELISA Kit, Pleasanton, U.K.) using the manufacturer's protocols.

Statistical analysis

Statistical analysis was performed by SPSS version-13 software. Parametric data are expressed as means $\pm$ standard deviations. Statistical differences between the groups were evaluated by one way-ANOVA with subsequent application of the Bonferroni test for post-hoc analysis. Differences yielding *p* values less than 0.05 were considered statistically significant.

Results

All study procedures were conducted in compliance with the European Directive 2010/63E4 guidelines ("Understanding Animal Research", November 2010), after receiving approval of the local ethics committee for animal experimentations (permit number 47/2012).

Metanephric cell isolation and handling:

Cells were isolated from fetal rat metanephroi, all cultures demonstrated excellent anchorage to the culture vessels and heavily stained for Oct-4 and SSEA-4, the specific markers for undifferentiated embryonic cells.

Fig. 1. Number of viable vs. apoptotic cells. Podocytes were exposed to high hydrostatic pressure, pellets detached from the flask bottoms during the 24h-incubation were differentially counted, viable vs. dead cells. Pressure enforcement significantly augmented detachment of both viable and apoptotic cells. Application of AT1 Angiotensin-II receptor blockers almost completely abolished the apoptotic detachment and viable podocyte shedding. Blocking the AT2 receptor almost completely abolished the apoptotic podocyte shedding but was less effective in preventing viable podocyte loss. Exposure to Angiotensin-II yielded a detachment of viable podocytes vs. shedding of apoptotic cells. The application of AT1 Angiotensin-II receptor blockers almost completely abolished the apoptotic podocyte shedding. However, it was less effective in preventing the loss of viable podocytes. The application of AT2 Angiotensin-II receptor blockers almost completely abolished the apoptotic podocyte shedding and was less effective in preventing loss of viable podocytes. Exposure to TGFβ₁ yielded a massive and significant detachment of both viable podocytes and apoptotic ones. However, TGFβ₁ receptor blocker almost completely abolished apoptotic podocyte shedding. Exposure to conditioned medium collected from pressure-subjected mesangial cells also resulted in significant shedding of both viable and apoptotic podocytes. AT1/AT2 Angiotensin-II receptor blockers abolished the apoptotic podocyte shedding. 1A. Podocyte cell count following pressure vs. Angiotensin-II, TGFβ₁ or mesangial cell medium (MCM) application. 1B. Podocyte cell count following pressure, Angiotensin-II, TGFβ₁ or receptor blockade. 1C. Cell count following MCM and Angiotensin-II or the TGFβ₁ receptor blockade. Abbreviations: Control – untreated podocyte cultures; cells not subjected to high hydrostatic pressure; Pressure – podocytes were exposed to high hydrostatic pressure for 1h; Podocytes in MCM – instead of pressure exposure, podocytes were cultured in conditioned medium of mesangial cells pre-exposed to pressure; Angiotensin-II – instead of pressure exposure, excess Angiotensin-II was added to podocyte culture medium; TGFβ₁ – instead of pressure exposure, excess TGFβ₁ was added to podocyte culture medium; AT1 blockade – Cells exposed to Losartan, an AT-1 receptor blocker 2h prior to the application of high hydrostatic pressure; AT2 blockade – Cells exposed to PD123319, an AT-2 receptor blocker 2h prior to the application of high hydrostatic pressure* – cell count significantly different from that of the respective control (P < 0.05).

Fig. 2. Podocin expression by podocytes. 2A. Podocin expression following pressure exposure vs. application of Angiotensin-II, TGFβ₁ or Mesangial cell medium (MCM). 2B. Podocin expression following pressure enforcement Angiotensin-II or receptor blockade. Podocin expression on cells that remained attached to the culture vessels were measured by flow cytometry. Almost All the untreated control podocytes expressed podocin. Applying pressure, TGFβ₁ or adding Angiotensin-II significantly decreased counts of podocin-expressing cells. The proportion of cells that expressed podocin remained unchanged in podocytes that were maintained in medium collected from mesangial cells. Pretreatment with an AT1 receptor blocker prior to pressure application abolished this decrease. However, pretreatment with an AT2 receptor blocker did not improve the pressure-exposed decrease. Exposure to Angiotensin-II significantly reduced podocin expression and applying AT1 Angiotensin-II receptor blockers almost completely abolished the podocin decrease. Exposure to an AT2 blocker abolished the podocin decrease. Abbreviations: Control – untreated podocyte cultures; cells not subjected to high hydrostatic pressure; Pressure – podocytes were exposed to high hydrostatic pressure for 1h; Podocytes in MCM – instead of pressure exposure, podocytes cultured in conditioned medium of mesangial cells pre-exposed to pressure; Angiotensin-II – instead of pressure exposure, excessive Angiotensin-II was added to podocyte culture medium; TGFβ₁ – instead of pressure exposure, excessive TGFβ₁ was added to podocyte culture medium. AT1 blockade – Cells exposed to Losartan, an AT-1 receptor blocker 2h prior to the application of high hydrostatic pressure; AT2 blockade – Cells exposed to PD123319, an AT-2 receptor blocker 2h prior to the application of high hydrostatic pressure; * - cell count significantly different from that of the respective control (P < .05).

Fig. 3. Integrin- β_1 expression by podocytes. 3A. Integrin- β_1 expression following pressure exposure vs. application of Angiotensin-II, TGF β_1 or MCM (mesangial cell medium). 3B. Integrin β_1 expression following pressure exposure enforcement Angiotensin-II or TGF β_1 receptor blockade. Integrin β_1 expression on cells that remained attached to the culture vessels were measured by flow cytometry analysis. Almost All the untreated control podocytes expressed β_1 - integrins on podocyte membranes. Pressure application, exposure to Angiotensin-II, and to TGF β_1 , significantly reduced integrin β_1 expression. The proportion of β_1 -containing integrin expressing cells was not altered in podocytes that were maintained in medium collected from mesangial cells. In podocytes directly exposed to pressure, blocking AT1/AT2 receptors resulted in integrin β_1 expression that did not statistically differ from that of controls. In Angiotensin-II exposed podocytes, AT1 blocking resulted in a significant inhibition of integrin β_1 expression, whereas no inhibition was seen following AT2 receptor blocking. Abbreviations: Control – untreated podocyte cultures; cells not subjected to high hydrostatic pressure; Pressure – podocytes were exposed to high hydrostatic pressure for 1h; Podocytes in MCM – instead of pressure exposure, podocytes cultured in conditioned medium of mesangial cells pre-exposed to pressure; Angiotensin-II - instead of pressure exposure, excessive Angiotensin-II was added to podocyte culture medium; TGF β_1 - instead of pressure exposure, excessive TGF β_1 was added to podocyte culture medium. AT1 blockade – Cells exposed to Losartan, an AT-1 receptor blocker 2h prior to the application of high hydrostatic pressure; AT2 blockade – Cells exposed to PD123319, an AT-2 receptor blocker 2h prior to the application of high hydrostatic pressure; * - cell count significantly differs from that of the respective control ($p < .05$).

Fig. 4. Production of TGFβ₁ by podocytes and mesangial cells. TGFβ₁ synthesis were measured in podocyte and mesangial cells supernatants by specific ELISA assays. Control podocytes released low amounts of TGFβ₁ to the culture medium. Podocytes subjected to high hydrostatic pressure or exposed to Angiotensin-II, produced similar low TGFβ₁ concentrations. Only when placed in media of mesangial cells pre-exposed to pressure, did podocytes demonstrate a fivefold increase in TGFβ₁ production. Mesangial cells reacted to pressure by augmenting TGFβ₁ synthesis by more than threefold and when podocytes were placed in the same milieu, we observed a synergistic augmentation of TGFβ₁ production. Abbreviations: Control – untreated podocyte cultures; Pressure – podocytes were exposed to high hydrostatic pressure for 1h; Podocytes in MCM (mesangial cell medium) – instead of pressure exposure, podocytes were cultured in conditioned medium of mesangial cells pre-exposed to pressure; Angiotensin-II – instead of pressure exposure, excess Angiotensin-II was added to podocyte culture medium; TGFβ₁ – instead of pressure exposure, excess TGFβ₁ was added to podocyte culture medium. * – cell count significantly differs from that of the respective control (P < .05); MC Pressure – mesangial cells were placed in a pressure-loading apparatus for 1h to mimic the incidence of malignant hypertension.

Metanephric cell differentiation: Progressive loss of the undifferentiated embryonic cell phenotype:

In control cells, remaining in their original Knock-Out™ DMEM medium throughout the entire experiment, expression of undifferentiated embryonic cell markers Oct-4 and SSEA-4 was evident at day 4 and persisted throughout the entire study. No unspecific staining for markers characteristic of differentiating cells, was observed. By contrast, cells placed in medium selective for podocyte or mesangial cell differentiation, demonstrated progressive loss of Oct-4 and SSEA-4 expression (data not shown).

Gradual appearance of differentiated podocyte or mesangial cell markers

Concomitantly with progressive loss of the embryonic cell phenotype, immunohistochemical staining for podocyte markers gradually appeared on the cell surfaces.

Cells grown in podocyte selective medium had unmistakable podocyte appearance and stained for WT-1, keratin, nephrin, PDGF-β receptors and synaptopodin. Concomitantly, quantitative RT-PCR demonstrated high levels of synaptopodin, podocalyxin and ENPEP gene expression. Finally, high percentages of cells positive for ZO1, podocin were confirmed by flow cytometry.

It is noteworthy that podocyte differentiation took less time than that of mesangial cells. Disappearance of Oct-4 and SSEA-4 and the appearance of profound staining for α-SMA, Thy-

Fig. 5. Production of Angiotensin-II by podocyte and mesangial cells. Angiotensin-II synthesis were measured in podocyte and mesangial cells supernatants by specific ELISA assays. Angiotensin-II synthesis by podocytes was initially low and not altered by exposure to TGFβ₁ or exposure to culture medium collected from mesangial cells pre-exposed to pressure. This changed very modestly, although statistically significantly, with pressure application. In mesangial cell cultures, Angiotensin-II production was greatly increased by exposure to pressure. However, no synergistic effect on Angiotensin-II synthesis was elicited in podocytes placed in media of pressure-exposed mesangial cells.

1, and PDGF-β receptors, the markers of differentiated mesangial cells, was evident only starting day 45.

Similar to previously published data, following 60 days of culture, the cells no longer expressed any markers of undifferentiated embryonic cells [1]. Instead, markers characteristic of either differentiated podocyte or mesangial cell phenotypes were fully expressed.

Differential count of detached podocytes following application of high hydrostatic pressure, Angiotensin-II, TGFβ₁ or receptor blockers

Culture medium collected from podocytes that were never exposed to pressure contained negligible amounts of detached viable ($2200 \pm 1500/\text{ml}^3$) and apoptotic ($1500 \pm 800/\text{ml}^3$) podocytes. Pressure enforcement significantly augmented detachment of both viable ($14300 \pm 7700/\text{ml}^3$) and apoptotic ($7240 \pm 4700/\text{ml}^3$) cells ($p < .05$) (Fig. 1A).

Exposure to pressure together with the application of AT1 Angiotensin-II receptor blockers almost completely abolished the apoptotic detachment ($1660 \pm 2080/\text{ml}^3$) and viable podocyte shedding ($2000 \pm 2640/\text{ml}^3$). Blocking the AT2 receptor almost completely abolished the apoptotic podocyte shedding ($3000 \pm 2000/\text{ml}^3$) but was less effective in preventing viable podocyte loss ($8000 \pm 2500/\text{ml}^3$). The application of TGFβ₁ receptor blocker had no inhibitory effect on the detachment of viable ($18400 \pm 2600/\text{ml}^3$) or apoptotic podocytes ($17300 \pm 4840/\text{ml}^3$) ($P < .001$) following high hydrostatic pressure application (Fig. 1B).

Exposure to Angiotensin-II yielded a detachment of $6300 \pm 3020/\text{ml}^3$ viable podocytes vs. shedding of $7900 \pm 2410/\text{ml}^3$ apoptotic cells (Fig. 1a). The application of AT1 Angiotensin-II receptor blockers almost completely abolished the apoptotic podocyte shedding ($2000 \pm 1000/\text{ml}^3$). However, it was less effective in preventing the loss of viable podocytes ($5000 \pm 1500/\text{ml}^3$) (Fig. 1b). The application of AT2 Angiotensin-II receptor blockers almost completely abolished the apoptotic podocyte shedding ($1000 \pm 500/\text{ml}^3$) and was less effective in preventing loss of viable podocytes ($5000 \pm 2000/\text{ml}^3$) (Fig. 1B).

The replacement of Angiotensin-II by TGF β_1 yielded a massive and significant detachment of both viable podocytes ($19700 \pm 7100/\text{ml}^3$) and apoptotic ($18500 \pm 3500/\text{ml}^3$) ones ($p < .001$), similar to that seen by pressure enforcement. (Fig. 1a). Blocking the TGF β_1 receptor had no inhibitory effect on viable podocyte detachment ($13000 \pm 8480/\text{ml}^3$). However, the TGF β_1 receptor blocker almost completely abolished apoptotic podocyte shedding ($3500 \pm 700/\text{ml}^3$) (Fig. 1B).

Exposure to conditioned medium collected from pressure-subjected mesangial cells also resulted in significant shedding of both viable ($12900 \pm 7800/\text{ml}^3$) and apoptotic podocytes ($13600 \pm 7100/\text{ml}^3$, $P < .001$) (Fig. 1a). However, the detachment rate decreased compared to that elicited by pressure or by TGF β_1 . AT1/AT2 Angiotensin-II receptor blockers abolished the apoptotic podocyte shedding ($1800 \pm 1000/\text{ml}^3$ vs. $1500 \pm 1400/\text{ml}^3$ respectively). However, the AT1/AT2 receptor blockade was less effective in preventing loss of viable podocytes ($4300 \pm 2500/\text{ml}^3$ vs. 3500 ± 2400 respectively). The TGF β_1 receptor blocker had no inhibitory effect on the detachment of viable ($11300 \pm 1860/\text{ml}^3$) or apoptotic podocytes ($12000 \pm 2640/\text{ml}^3$) (Fig. 1c).

Expression of transmembrane adhesion proteins regulating podocyte detachment

We then performed experiments on cells that remained attached to the culture vessels. We examined whether applying pressure or alternatively, exposure to Angiotensin-II or TGF β_1 will affect the attached podocyte count and their integrin and/or podocin expression.

Almost all the untreated control podocytes expressed podocin ($84.8 \pm 15.6\%$). Applying pressure or adding TGF β_1 significantly decreased counts of podocin-expressing cells ($54.3 \pm 6\%$ $p = .01$ and $56.7 \pm 3.25\%$, $p = .01$ respectively) (Fig. 2a). Exposure to Angiotensin-II yielded $61.5 \pm 6.7\%$ ($p = .03$) podocin expressing cells. The proportion of cells that expressed podocin remained unchanged in podocytes that were maintained in medium collected from mesangial cells ($87.6 \pm 13.8\%$) (Fig. 2A).

Pretreatment with Losartan (an AT1 receptor blocker) prior to pressure application abolished this decrease ($92.2 \pm 7\%$). However, pretreatment with PD123319 (an AT2 receptor blocker) did not improve the pressure-exposed decrease of podocin ($57.4 \pm 0.92\%$) (Fig. 2B). Exposure to Angiotensin-II significantly reduced podocin expression ($61.5 \pm 6.7\%$) and applying AT1 Angiotensin-II receptor blockers almost completely abolished the podocin decrease ($90.2 \pm 4.97\%$) (Fig. 2B). Exposure to an AT2 blocker abolished the podocin decrease ($84.7 \pm 1.6\%$).

We also estimated the expression of β_1 -containing integrins on podocyte membranes, since its expression is critical for maintaining the structural integrity of the glomerulus. Of the control podocytes, $56 \pm 10\%$ expressed integrin β_1 . Pressure application, exposure to Angiotensin-II, and to TGF β_1 , significantly reduced integrin β_1 expression ($30.9 \pm 13\%$ $p < .02$, $28 \pm 8.8\%$ $p < .01$ and $18 \pm 8\%$ $p < .001$, respectively) (Fig. 3A). The proportion of β_1 -containing

Fig. 6. Schematic summary of glomerular injury process due to malignant hypertension.

integrin expressing cells was not altered in podocytes that were maintained in medium collected from mesangial cells ($53.2 \pm 30\%$).

In podocytes directly exposed to pressure, blocking AT1 receptors resulted in integrin β_1 expression that did not statistically differ from that of controls ($41 \pm 13\%$). The same was true for blocking AT2 receptors ($50.2 \pm 6.1\%$). In Angiotensin-II exposed podocytes, AT1 blocking resulted in a significant inhibition of integrin β_1 expression ($40.3 \pm 21\%$), whereas no inhibition was seen following AT2 receptor blocking ($26.2 \pm 4.9\%$) (Fig. 3B).

Synthesis of TGF β_1 and Angiotensin-II by podocytes and mesangial cells subjected to high hydrostatic pressure

Control podocytes released low amounts of TGF β_1 to the culture medium (20.3 ± 2.5 pg/ml). Podocytes subjected to high hydrostatic pressure or exposed to Angiotensin-II, produced similar low TGF β_1 concentrations (20.5 ± 4.5 pg/ml and 21.9 ± 3.5 pg/ml, respectively). Only when placed in media of mesangial cells pre-exposed to pressure, did podocytes demonstrate a fivefold increase in TGF β_1 production (125 ± 19 pg/ml $p < .001$). It is noteworthy to mention that mesangial cells reacted to pressure by augmenting TGF β_1 synthesis by more than threefold (58 ± 13 pg/ml $p < .03$) (Fig. 4) and when podocytes were placed in the same milieu, we observed a synergistic augmentation of TGF β_1 production (125 ± 19).

Angiotensin-II synthesis by podocytes was initially low (250 ± 50 pg/ml) and not altered by exposure to TGF β_1 (314 ± 59 pg/ml) or exposure to culture medium collected from mesangial cells pre-exposed to pressure (342 ± 21) (Fig. 5). This changed very modestly, although statistically significantly, with pressure application (367 ± 14 pg/ml $p = .02$). In mesangial cell cultures, Angiotensin-II production was greatly increased by exposure to pressure (2999 ± 580 pg/ml $P < .001$). However, no synergistic effect on Angiotensin-II synthesis was elicited in podocytes placed in media of pressure-exposed mesangial cells (342 ± 21) (Fig. 5).

Discussion

Extensive loss of podocytes from the GBM signifies severe renal insult and progression towards end-stage renal failure [4-7]. Using an *in vitro* model of pressure-induced malignant hypertension, we have previously shown that mesangial cells play a deleterious role in orchestrating the acute renal tissue insult that is associated with malignant hypertension [1, 24, 25]. In this study, we focused on the podocytes. We found that the magnitude of malignant hypertension-induced podocyte damage depends on the amount of Angiotensin-II involved. Podocyte detachment can be induced by both autocrine and paracrine effects. High pressure induces podocytes to increase Angiotensin-II production, a direct autocrine response, and via AT1 receptors to structural changes in the manifestation of adhesion proteins, culminating in viable podocyte detachment. In addition, the adjunct mesangial cells respond to pressure by further/higher increased Angiotensin-II and TGF- β_1 production, a paracrine effect, culminating in massive apoptosis and detachment of non-viable podocytes.

In a study done by Ruiz-Ortega et al., it was indicated that Angiotensin II via AT1 receptors regulates extracellular matrix (ECM) accumulation mediated by the endogenous production of profibrotic growth factors, such as TGF- β [22]. In our previous study, it was found that Angiotensin-II is excessively secreted by mesangial cells subjected to pressure [1]. The present results extend this observations, and demonstrate that TGF β_1 secretion is also increased in mesangial cells in addition to Angiotensin-II. While excessive Angiotensin-II initiates podocyte apoptosis, TGF β_1 is responsible for the loss of viable and apoptotic podocytes. Podocytes respond to TGF β_1 stimulation in a concentration dependent manner with high concentrations of TGF β_1 causing podocyte apoptosis [32]. Angiotensin-II is also involved in viable podocyte detachment. Augmented Angiotensin-II results in decreased expression of podocin and integrin β_1 , proteins that are both vital in viable podocytes

adhesion to the GBM and in the interaction of podocytes with other GBM components.

Podocyte detachment from the GBM initiates and accompanies the majority of renal glomerular involved diseases [4-7, 33-35]. Most detached podocytes survive and reach the urinary tract as viable cells, retaining the ability to attach to a culture vessel *in vitro* [36-39]. In a number of chronic renal conditions, the amount of viable podocytes excreted in urine predicts the magnitude of disease progression [33, 40]. To the best of our knowledge, this is the first study on podocyte detachment mechanisms in a cellular simulation of malignant hypertension.

Apoptotic death vs. loss of viable podocytes

In the present study, podocytes responded to *in vitro* induced malignant hypertension by massively detaching from their culture vessels. Some of the detached cells were irreparably apoptotic. Yet about half of the cells were viable and capable of *de novo* attachment and growth in a new vessel supplied with fresh culture medium. Exposure to exogenous Angiotensin-II as an alternative to pressure yielded moderately augmented detachment of apoptotic as well as viable podocytes. Angiotensin-II induces podocyte apoptosis by translocating NFκB/p65 and decreasing TRPC6 channel activity in ADR-treated podocytes [41, 42]. One study has shown that the reduction of albuminuria by olmesartan (an angiotensin II receptor blocker) treatment is associated with increased number of podocytes and a decreased number of apoptotic glomerular cells in diabetic glomeruli [43]. In the present study, blocking the AT1 receptor attenuated both apoptotic and detached of viable podocytes. To a lesser extent, detachment of viable podocytes was exerted via the AT2 receptor.

Unlike exposure to Angiotensin-II, exposure to TGFβ₁ yielded severe detachment of both viable and apoptotic podocytes. This is also the case in comparison to the effect elicited by pressure.

Significant efforts have been made to inhibit TGF-β's action, with the aim of decreasing the progression of renal fibrosis. Over the years, several novel approaches have been tested, which include inhibiting, neutralizing and blocking TGF-β expression [27, 28, 44]. In our experiments, blocking the TGFβ₁ receptor prevented detachment and loss of apoptotic podocytes, but was less effective in preventing viable podocyte loss. This is an indication that the TGFβ₁ receptor machinery is not involved in the TGF-regulated loss of viable podocytes. However, TGF receptors are evidently involved in podocyte apoptosis, since their blockade effectively counteracted the receptor-based mechanisms of Angiotensin-II modulated detachment of apoptotic podocytes.

Synthesis of TGFβ₁

Mesangial cells reacted to pressure enforcement by a threefold increase in TGFβ₁ synthesis. Podocytes however, were not directly triggered by pressure application to secrete TGFβ₁. Only when podocytes were exposed to media of mesangial cells subjected to pressure, did they demonstrate enhanced TGFβ₁ secretion.

Expression of transmembrane adhesion proteins

In an attempt to uncover the mechanisms of malignant hypertension-triggered podocyte detachment, we investigated changes in expression of podocin and integrinβ₁, the main structural adhesion proteins responsible for podocytes attaching to the glomerular filtration barrier.

Podocin was expressed by almost all untreated control podocytes. However, Podocin expression was significantly decreased in cells directly exposed to pressure, Angiotensin-II or TGFβ₁, compared to untreated controls. Podocin expression remained unchanged only in podocytes maintained in media of mesangial cells pre-exposed to pressure. Blocking of AT1 and not AT2, prevented the expected pressure decreased podocin expression. This indicates that Angiotensin-II exerts its effect on podocin via the AT1 receptor. Thus, podocin appears to play an important role in podocyte loss after the incidence of malignant hypertension.

We further estimated the effects of the protein integrin β_1 on podocytes, since *in vivo* expression of this protein is crucial for the preservation of the glomerular structural integrity [35]. Integrin β_1 mediates podocyte adhesion, migration, signal transduction and the assembly of the extracellular matrix. The best characterized integrin β_1 ligand is fibronectin, which actively participates in many adhesive processes that lead to signal transduction [35]. In our experiments, we found that pressure as well as Angiotensin-II or TGF β_1 , significantly reduced integrin β_1 expression. In contrast, pressure-exposed mesangial cell media did not decrease integrin β_1 expression. Blocking of Angiotensin-II receptors in podocytes exposed to high pressure had little effect. Yet, when the podocytes were directly exposed to exogenous Angiotensin-II instead of pressure, they demonstrated inhibition of integrin β_1 expression, mediated via AT1 but not AT2 receptors. Taken collectively, we see that excessive Angiotensin-II affects integrin β_1 expression in podocytes.

Our results suggest that following malignant hypertension-induced acute renal injury, mesangial cells are fully accountable for podocyte loss. Mesangial cells release excessive Angiotensin-II and TGF β_1 in response to pressure. Podocytes, in turn, respond to enhanced Angiotensin-II by decreasing podocin and integrin β_1 expression, both mediated via the AT1 receptor, leading to detachment (Fig. 6).

Conclusion

Malignant hypertension induces podocyte detachment by both autocrine and paracrine effects. Podocytes increase Angiotensin-II production in a direct autocrine response. This leads, via AT1 receptors, to structural changes in the manifestation of adhesion proteins, culminating in viable podocyte detachment. Adjunct mesangial cells via a paracrine effect, respond to pressure by increasing Angiotensin-II and TGF- β_1 production, leading to massive apoptosis and detachment of non-viable podocytes.

Our results indicate that podocyte injury plays an important role in the pathogenesis of malignant hypertension-induced kidney injury, and suggest that podocytes are potentially an important therapeutic target in hypertensive renal disease.

Disclosure Statement

No conflict of interests existing.

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