Enhanced angiotensin II production by renal mesangium is responsible for apoptosis/proliferation of endothelial and epithelial cells in a model of malignant hypertension
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Objective The systemic renin–angiotensin system (RAS) plays a crucial role in the pathogenesis of malignant hypertension. However, the intrarenal RAS might be at least equally important. We investigated the relationship between intrarenal RAS and mesangial, epithelial and endothelial cell proliferation/apoptosis in a model of malignant hypertension.

Methods Cultured murine mesangial cells were subjected to 160 mmHg hydrostatic pressure for 1 h. Angiotensin II was assessed by radio-immunoassay (RIA); pro-metalloproteinase-1 (pro-MMP-1) by enzyme-linked immunosorbent assay (ELISA); hydrogen peroxide (H₂O₂) by photocolorimetric assay, apoptosis by terminal dUTP (2-deoxyuridine 5'-triphosphate) nick-end labelling (TUNEL), p53 by western blot and proliferation by [3H]thymidine incorporation, with or without angiotensin II and/or angiotensin II type 1/angiotensin II type 2 (AT-1/AT-2) receptor blockers. Endothelial and epithelial cells were similarly treated, and the same parameters evaluated. Further, untreated cells of both lines were cultured in conditioned medium of mesangial cells exposed to pressure. Their proliferation, apoptosis and angiotensin II production were also assessed.

Results High hydrostatic pressure increased angiotensin II production by mesangial cells, coinciding with augmented apoptosis and proliferation. Co-stimulation with exogenous angiotensin II amplified both effects. Pressure per se evoked no response in endothelial/epithelial cells, while exogenous angiotensin II stimulated proliferation and apoptosis. No augmentation of p53 expression was evident. These effects were abolished by anti-angiotensin-II peptide, saralasine and losartan, but not by PD123319. Incubation of untreated cells in medium of mesangium subjected to pressure, augmented proliferation and apoptosis. No significant changes were noticed in pro-MMP or H₂O₂.

Conclusions Mesangium plays a deleterious role in the pathogenesis of malignant hypertension. High hydrostatic pressure stimulates angiotensin II synthesis by mesangial cells. The latter is responsible for hypercellularity and apoptotic death of mesangial, endothelial and epithelial cells. In this model, exaggerated apoptosis and proliferation are mediated via the angiotensin II pathway independently of p53 gene activation. J Hypertens 25:1041–1052 © 2007 Lippincott Williams & Wilkins.

Keywords: angiotensin II, apoptosis, endothelium, epithelium, malignant hypertension, p53, proliferation, renal mesangium, renin–angiotensin system

Introduction Hypertensive emergencies are acute, life-threatening events associated with a marked increase in blood pressure [1]. Systemic as well as local renin–angiotensin systems (RAS) are inevitably involved, playing a crucial role in the pathogenesis of the disease [2]. In the context of malignant hypertension, intrarenal RAS activation is undoubtedly the most detrimental. Intrarenal angiotensin II levels are about 1000 times higher compared to systemic angiotensin II concentrations in a normal state, and are elevated further following the onset of hypertension [3]. Normally, elevation of systemic blood pressure per se would suppress overproduction of renin via a feedback inhibition mechanism [4]. However, in the case of malignant hypertension this regulatory mechanism is impaired and exaggerated renin production and, consequently, RAS activation go on undisturbed. At the cellular level, the nature of biochemical cascade initiated by an event of malignant hypertension and the resultant irreversible renal tissue damage are yet to be elucidated.

The kidney glomerular capillary network is unique in its inner structure. The wall of glomerular capillaries consists of two distinct compartments: a peripheral part filling out the Bowman’s space and covered by epithelial cells, basement membrane and endothelial cells, and a...
central part, comprising mesangial cells and the adjoining endothelial cells [5]. The myocyte-like mesangial cells are capable of contracting in response to pressure enforcement, altering the effective renal glomerular surface area and thus regulating the glomerular filtration rate of the kidney. By this autoregulatory mechanism, the renal mesangial core controls the haemodynamic changes in intrarenal capillaries and counterbalances fluctuations of systemic blood pressure [6–8]. Accordingly, under normal conditions both mesangial and endothelial cells are only exposed to low hydrostatic pressure, approximately 50% of mean systemic arterial pressure [9]. By contrast, in the case of malignant hypertension the autoregulatory function of the renal mesangial core is totally lost. As a result, intrarenal pressure renders levels equal to systemic blood pressure, bringing about deleterious renal tissue damage.

Thus far, however, the direct effects of malignant hypertension and the resultant RAS activation on specific manifestations of renal tissue damage, such as hypercellularity, production of inflammatory mediators or apoptotic cell death, have never been systematically approached. The aim of the present study was to investigate the relationship between malignant hypertension and apoptosis and/or proliferation of renal mesangial, epithelial and endothelial cells. The expression of p53, the regulator of apoptosis in various cell types, as well as production of angiotensin II, the specific stimulator of renal cell apoptosis and proliferation, were assessed concomitantly.

Materials and methods

Animals
Mesangial cells were isolated from renal glomerular cortices of 2-month-old healthy C57B and p53 knockout murine strains. The animals were purchased from Harlan Laboratories (Houston, Texas, USA) and, prior to sacrifice by halothane overdose, were maintained for 1 week under specific pathogen-free conditions in the local animal facilities, according to the NIH Guide for the care and use of laboratory animals [10]. The experimental protocol received approval of the local ethics committee for animal experimentation.

Cell cultures

Mesangial cell isolation and culture
The cells were purified, identified and subcultured in a highly specific selective medium, RPMI 1640 with d-valine substituted for L-valine and supplemented with 20% fetal calf serum, as described elsewhere [11–13].

Epithelial cell culture
A renal epithelial cell line, KNRK, was purchased from ATCC (American Type Cell Collection, Manassas, Virginia, USA) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), according to the manufacturer’s protocols, as described elsewhere [11].

Endothelial cell culture
A human umbilical vein endothelial cell line (HUVEC) was purchased from PromoCell company (Heidelberg, Germany) and cultured in a specific low serum (2% FCS) medium according to the manufacturer’s instructions.

Pressure-loading apparatus
The pressure-loading apparatus consisted of a water bath supplied with a heating device and a pressure chamber placed into the water preheated to 37°C. Constant air pressure within the chamber (160 ± 3 mmHg, 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 at 0.5 l/min rate. The air flow consisted of 20% O2 and 5% CO2. The temperature inside the pressure chamber was maintained at 37 ± 0.2°C by a thermostat placed within the water bath.

Experimental design

Stage A: Preliminary studies
Tissue culture flasks of 50 ml capacity, containing subconfluent monolayers of either mesangial, epithelial or endothelial cells, were subjected to 80, 120, 150, 160 or 180 mmHg hydrostatic pressure for 30 min, 1 h or 2 h in a pressure-loading apparatus.

Since maximal proliferative and apoptotic responses to pressure were obtained following incubation for 1 h at ≥150 mmHg, and since no significant differences were observed between the 150, 160 and 180 mmHg, we chose the 1-h, 160 mmHg pressure application regimen for the present experimental setting.

Stage B: Pressure application
The cells were subjected, or not, to 160 mmHg hydrostatic pressure in the pressure chamber for 1 h in the presence or absence of the following:

1. 160 mmHg pressure, no additions to the medium;
2. 10⁻⁸ mol/l angiotensin II (A-II);
3. 10⁻⁶ mol/l losartan (angiotensin II type 1 (AT-1) receptor blocker);
4. 10⁻⁶ mol/l PD123319 (angiotensin II type 2 (AT-2) receptor blocker);
5. 10⁻⁶ mol/l saralasin (a ‘universal’ A-II receptor blocker);
6. 10⁻⁸ mol/l antibody to angiotensin II;
7. 10⁻⁶ mol/l captopril (A-II converting enzyme inhibitor).

Preliminary dose–response curve studies have been performed for this as well as for some previous investigations...
[12,13], using angiotensin II concentrations from $10^{-14}$ mol/l to $10^{-4}$ mol/l. They demonstrated that cells responded to angiotensin II stimulation by augmented apoptosis and proliferation at $10^{-8}$ mol/l, and no appreciable additional responses of this nature could be elicited by higher angiotensin II concentrations. The blocking agents, when applied to angiotensin II-treated cultures, were added 2 h prior to the latter, all the concentrations also being chosen based on the dose–response curves from preliminary experiments and/or our previous studies.

**Stage C: Application of conditioned medium**

Twenty-four hours following pressure enforcement, the conditioned media from mesangial cells subjected to pressure, or to pressure combined with angiotensin II addition to the medium, were collected. Subsequently, three new portions of endothelial or epithelial cells, never subjected to pressure, were cultured for 48 h as follows:

1. placed in conditioned medium from control mesangial cells not subjected to pressure;
2. placed in conditioned medium from mesangial cells exposed to high hydrostatic pressure prior to the medium collection;
3. placed in conditioned medium from mesangial cells stimulated with $10^{-8}$ mol/l angiotensin II prior to pressure application.

Following 1 h in the pressure chamber (Stage B), or 48 h incubation in conditioned media (Stage C), all the cell media were discarded, the monolayers washed repeatedly in phosphate-buffered saline (PBS), trypsinized in 25% trypsin–EDTA solution and, following three additional washings in PBS, the cells were transferred in equal aliquots to 12-well tissue culture plates, onto 18-mm round coverglass slips inserted within the plate wells. The cells were maintained in a humid incubator with 5% CO$_2$ at 37°C for 48 h, to be used in the following experiments.

**Proliferation assays**

DNA synthesis was evaluated by $[^3]$H]thymidine incorporation.

**Apoptosis assays**

These were performed by two independent methods, terminal dUTP (2-deoxyuridine 5’-triphosphate) nick-end labelling (TUNEL) assay and Mayer haematoxylin staining followed by visual microscopic examination and differential count of apoptotic cells, as described elsewhere [11–13]. In brief, 10 microscopic fields were chosen at random from each microscope slide and 100 cells per microscopic field were then randomly counted, comprising a differential count of the bright-brown, horseradish peroxidase-stained apoptotic cells. Percent apoptosis per slide was calculated as described previously [12,13], by summarizing the differential count of apoptotic cells out of a total of 1000 cells counted in each slide, as follows:

$$\% \text{Apoptosis} = \frac{\text{ACC} \times 100\%}{\text{TCC}}$$

where ACC is apoptotic cell count; TCC, total cell count; and ACC / TCC, number of apoptotic cells out of the total cell count, expressed as a percentage.

**Expression of p53 protein**

Expression of p53 was assessed by Western blot analysis using a slight modification of a method described elsewhere [14]. In brief, the cell cultures were washed repeatedly in PBS and lysed in TLB buffer consisting of 50 mmol/l Tris (pH 7.5), 100 mmol/l NaCl, 1% Triton X100, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulphate (SDS). Following total protein adjustment, equal amounts of protein sample buffer [50 mmol/l Tris, pH 6.8, 50% glycerol, 1% SDS, 0.75% dithiothreitol (DTT), 0.02% bromophenol blue] were added to each sample. The samples were boiled for 10 min and then loaded onto 10% SDS–polyacrylamide gel. The protein samples obtained were transferred to nitrocellulose membranes. A mixture of murine p53-specific monoclonal antibodies, Pab 242, Pab 248 and Pab 421, was used for binding to p53 protein. The p53 protein–antibody complexes were detected by horseradish peroxidase-conjugated secondary antibody, using the enhanced chemiluminescence system (Amersham, Bucks, UK). Cysplatin 3 (3 µg/ml) was used as an activating factor in a positive control, and Hp53 was applied as a constitutive p53 marker.

**Angiotensin II release**

Angiotensin II release by mesangial, epithelial and endothelial cells to their culture medium was evaluated by a competitive radio-immunoassay (RIA) (Bachem, Bubendorf, Switzerland), with high sensitivity (10 pg/ml per test tube) and a 100% recovery of the standard solution in the additional standard test procedure. According to the manufacturer, the anti-angiotensin II antibody used in this assay provided 100% cross-reactivity with angiotensin II, 0.5% cross-reactivity with angiotensin I, 0.9% cross-reactivity with renin and 0% cross-reactivity with ANP (atrial natriuretic peptide) or vasopressin.

**Pro-metalloproteinase-1 assay**

Pro-metalloproteinase-1 (Pro-MMP-1) was measured by an enzyme-linked immunosorbent assay (ELISA), using a mouse monoclonal antibody specific for pro-MMP-1, with 98–100% recovery range in cell culture media. According to the data provided by the manufacturer, the assay sensitivity was 0.021 ng/ml, assessment of intra- and interassay precision yielded mean coefficient of variability (CV) values of 4.5 and 9.4%, respectively, and no cross-reactivity with factors related to, or associated with, pro-MMP-1 was evident.
Evaluation of reactive oxygen species

Reactive oxygen species (ROS), as represented by hydrogen peroxide (H$_2$O$_2$), were evaluated by a quantitative hydrogen peroxide photoluminometric assay (Biotech, OXIS BIOTECH, Int, Inc., Portland, Oregon, USA) based on oxidation of ferrous ions (Fe$^{2+}$) to ferric ions (Fe$^{3+}$) by H$_2$O$_2$ under acidic conditions. The ferric ions bind to xylene orange dye, to form stable coloured complexes, and the magnitude of the colour can be measured subsequently in a spectrophotometer at 560 nm.

Statistical analysis

The results of each experimental variable are presented as means ± standard deviations of eight independent experiments (n = 8), while within a given experiment each variable has been performed in duplicate. The non-parametric Kruskal–Wallis test was applied to evaluate statistical differences between the results. Differences yielding P values less than 0.05 were considered statistically significant.

Results

Angiotensin II production assay

Angiotensin II synthesis was assessed in cell cultures that were not exposed to exogenous angiotensin II throughout the entire experiment. The results are summarized in Fig. 1. Exposure of mesangial cells from C57B mice to 160 mmHg pressure for 1 h resulted in a significant increase in angiotensin II production (29.08 ± 3.28 pg/mg cell protein versus control 20.55 ± 1.86 pg/mg cell protein, P < 0.009). Captopril addition to mesangial cell medium prior to pressure application prevented the increase in angiotensin II synthesis (18.24 ± 2.97 pg/mg cell protein versus control 20.55 ± 1.86 pg/mg cell protein, P = 0.076).

Mesangial cultures from p53 knockout mice demonstrated high basal angiotensin II synthesis (29.1 ± 3.1 pg/mg cell protein). Following pressure application it increased further (33.03 ± 5.17 pg/mg cell protein) and, similarly to C57B cultures, could be attenuated by captopril.

No significant basal angiotensin II synthesis was evident in either epithelial or endothelial cell cultures: the binding of radioactive tracer was within the range of non-specific binding (NSB) of the radio-immunoassay. Furthermore, 1 h exposure of either epithelial or endothelial cells to 160 mmHg pressure did not stimulate any detectable angiotensin II synthesis in these cultures (Fig. 1).

Proliferation assays

Proliferation of mesangial cell cultures exposed to high hydrostatic pressure for 1 h was significantly augmented compared to normal untreated controls (Fig. 2a; 3743 ± 952 cpm/mg cell protein versus 1159 ± 146 cpm/mg cell protein, P = 0.003). Stimulation with angiotensin II or angiotensin II combined with pressure application yielded an additional increase in proliferation rate (5542 ± 849 cpm/mg cell protein and 6606 ± 1074 cpm/mg cell protein, respectively, versus control 1159 ± 146 cpm/mg cell protein, P < 0.003 in both comparisons). Pretreatment of the angiotensin II stimulated cells with losartan (a specific AT-1 receptor blocker), or saralasin (a ‘universal blocker’ of all angiotensin II receptors), but not with PD123319 (a specific AT-2 receptor blocker) abolished the high pressure-induced enhancement of mesangial cell proliferation. Addition of either captopril or of a specific anti-angiotensin II antibody also resulted in abolishment of enhanced mesangial cell proliferation rate (1113 ± 106 and 1124 ± 98 cpm/mg cell protein, respectively, P > 0.05 compared to the basal 1159 ± 146 cpm/mg cell protein). In control p53 knockout mesangial cells the initially enhanced basal cell proliferation (1996 ± 659 cpm/mg cell protein) was, similarly to C57B cultures, augmented further following pressure application (2688 ± 530 cpm/mg cell protein).

By contrast, high pressure application evoked no increase in proliferative response of epithelial-like cell cultures (Fig. 2b; 1824 ± 248 versus control 1743 ± 159 cpm/mg cell protein, P > 0.05), while addition of angiotensin II to the medium significantly augmented their proliferative rate (5983 ± 908 cpm/mg cell protein, P = 0.005 compared to control). The effect of angiotensin II could be completely abrogated by blockade of the AT-1 receptor by losartan, reversing the radioactivity counts back to baseline levels. Blockade of AT-2 receptor by PD123319 was apparently less effective: the cell proliferation levels remained significantly higher compared to the baseline (3820 ± 850 versus basal 1743 ± 159 cpm/mg cell protein, P < 0.004). Still, the PD123319-modulated epithelial cell proliferation.
Proliferation rate was significantly decreased compared to that of cells treated with angiotensin II (3820 ± 850 versus 5983 ± 908 cpm/mg cell protein, \( P = 0.01 \)). Similarly, no excessive proliferation was observed in endothelial cell cultures following pressure application (Fig. 2c; 2683 ± 550 cpm/mg cell protein versus basal 2150 ± 450 cpm/mg cell protein, \( P < 0.05 \)).
Proliferation of fresh, untreated epithelial or endothelial cells was significantly enhanced following 48 h incubation in conditioned medium of mesangial cells previously subjected to high hydrostatic pressure, whether or not combined with angiotensin II treatment prior to pressure application.

**Apoptosis assays**

Mesangial cells subjected to high hydrostatic pressure demonstrated significantly elevated apoptotic rates compared to control cells (Fig. 3a; 22.2 ± 4.75% versus 0.9 ± 0.73%, \( P = 0.0001 \)) or to cells exposed to exogenously added angiotensin II (22.2 ± 4.75% versus

![Apoptosis assays](image-url)
9.0 ± 4.26%, P = 0.0007). Stimulation with angiotensin II combined with pressure application yielded a further increase in percent apoptosis (25.9 ± 9.25%, P < 0.005 compared with either control or angiotensin II-treated cells, 0.9 ± 0.73% and 9.0 ± 4.26%, respectively). The difference did not, however, reach statistical significance in comparison with percent apoptosis in cells subjected only to pressure application (i.e. 22.2 ± 4.75%). Blockade of AT-1 receptor with losartan significantly decreased percent apoptosis (18.3 ± 3.09% versus 25.9 ± 9.25%, P < 0.005 compared with either control or angiotensin II-treated cells, 0.9 ± 0.73% and 9.0 ± 4.26%, respectively). The difference did not, however, reach statistical significance in comparison with percent apoptosis in cells subjected only to pressure application (i.e. 22.2 ± 4.75%). Blockade of AT-1 receptor with losartan significantly decreased percent apoptosis (18.3 ± 3.09% versus 25.9 ± 9.25%, P = 0.04), while blockade of AT-2 receptor with PD123319 exerted no statistically significant effect on apoptosis of mesangial cells submitted to pressure (20.8 ± 5.45% versus 25.9 ± 9.25%, P = 0.08). Addition of captopril or anti-angiotensin II antibody prior to pressure application yielded apoptotic rates not statistically different from untreated controls (1.1 ± 0.39% and 1.25 ± 0.54%, P = 0.1 compared to basal 0.9 ± 0.73%). Basal apoptosis of mesangial cells from p53 knockout mice was augmented, and increased further following pressure application (26 ± 0.55% and 37 ± 0.71%, respectively, P = 0.003).

Unlike mesangial cells, the epithelial-like cell cultures subjected to high pressure exhibited apoptosis rates statistically not different from those of untreated controls (Fig. 3b; 2.75 ± 1.28% versus control 2.87 ± 1.64%, P = 0.07). Similarly, in endothelial cell cultures augmentation of apoptotic response was not elicited by pressure application (Fig. 3c).

Angiotensin II addition to epithelial, but not to endothelial cell cultures resulted in augmentation of apoptosis, irrespective of pressure application (12.37 ± 3.06% and 10.62 ± 3.20%, P = 0.001). Pretreatment with losartan or saralasin, or addition of anti-angiotensin II antibody significantly decreased the angiotensin II-induced apoptosis (1.62 ± 1.18%, 4.85 ± 2.03%, 4.74 ± 1.91%, respectively, versus angiotensin II induced 12.37 ± 3.06%, P = 0.0001), while addition of PD123319 proved ineffective (11.62 ± 2.92% versus 12.37 ± 3.06%, P = 0.07).

By contrast, when incubated for 48 h in conditioned medium of mesangial cells subjected to high pressure, the untreated epithelial cells demonstrated significantly augmented apoptosis, irrespective of co-stimulation with angiotensin II.

p53 expression
In all experimental situations, the p53 gene remained inactive in cultures subjected to pressure. This was true for mesangial as well as for epithelial and endothelial cells, as compared to a positive control whereby augmented p53 expression was induced in the same cells by incubation with 3 μg/ml cisplatin (Fig. 4).

Pro-MMP-1 determination
The highest basal pro-MMP-1 concentrations were found in HUVEC endothelial cell line culture media (0.072 ± 0.018 μmol/ml). They were significantly elevated...
compared to those found in either mesangial (0.049 ± 0.012 μmol/ml) or epithelial (0.027 ± 0.09 μmol/ml) cell cultures (Fig. 5a–c; P < 0.05 for all comparisons). High hydrostatic pressure enforcement, whether or not combined with angiotensin II treatment, did not result in significant alterations of pro-MMP-1 content in any cell culture. Similarly, application of angiotensin II receptor blockers, angiotensin-converting enzyme (ACE) inhibition by captopril or addition of anti-angiotensin II antibody did not produce any significant changes in this parameter.

Hydrogen peroxide formation

The results of H$_2$O$_2$ measurements are summarized in Fig. 6a–c. Basal H$_2$O$_2$ was 0.48 ± 0.13 μmol/ml in mesangial cells, 0.42 ± 0.15 μmol/ml in epithelial cells and 0.32 ± 0.12 μmol/ml in HUVEC (P = NS). No elevated H$_2$O$_2$ concentrations were detected in cultures of cells subjected to pressure. Application of angiotensin II receptor blockers, ACE inhibition by captopril or addition of anti-angiotensin II antibody did not change H$_2$O$_2$ content in cultures of mesangial and epithelial cell cultures (Fig. 5a–c; P < 0.05 for all comparisons). High hydrostatic pressure enforcement, whether or not combined with angiotensin II treatment, did not result in significant alterations of pro-MMP-1 content in any cell culture. Similarly, application of angiotensin II receptor blockers, angiotensin-converting enzyme (ACE) inhibition by captopril or addition of anti-angiotensin II antibody did not produce any significant changes in this parameter.

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cells. In endothelial cell cultures, inhibition of angiotensin II synthesis by captopril as well as prevention of angiotensin II entrance into the cells by either receptor blockers or antibody binding showed a tendency to decrease H$_2$O$_2$ formation. This decrease, however, did not reach statistical significance (0.33 ± 0.15 μmol/ml to 0.36 ± 0.15 μmol/ml, versus basal 0.41 ± 0.14 μmol/ml, $P = 0.6$).

**Discussion**

The aim of the present study was to investigate the relationship between application of high hydrostatic pressure and subsequent manifestations of renal tissue damage in an in-vitro model of severe malignant hypertension. For this purpose, angiotensin II synthesis, proliferation rates, apoptosis and expression of cell cycle regulating p53 gene were assessed separately in cultured renal mesangial, epithelial and endothelial cells subjected to high hydrostatic pressure. In renal mesangial cell cultures, application of high hydrostatic pressure resulted in significant augmentation of angiotensin II synthesis, along with increased proliferation and apoptosis. Stimulation with exogenously added angiotensin II prior to pressure enforcement brought about a synergistic effect, i.e. a further moderate increase of both apoptosis and proliferation. In contrast, no appreciable production of angiotensin II was evident in either epithelial or endothelial cell cultures, before or after pressure application. Concordantly, no appreciable changes in proliferation or apoptosis were observed in epithelial or endothelial cells following exposure to pressure per se. Exogenous angiotensin II or, alternatively, conditioned media of pressure-pretreated mesangial cells which has been shown to contain considerable amounts of endogenously produced angiotensin II, stimulated apoptosis and proliferation in epithelial, but not endothelial cells, irrespective of pressure enforcement.

Malignant hypertension is acute severe acceleration of systemic blood pressure generally exceeding 200/140 mmHg (mean arterial pressure 160 mmHg), culminating in target organ damage [1–2,14]. The most common clinical outcomes of malignant hypertension are encephalopathy, neuroretinopathy, papilloedema and/or renal tissue damage, brought about by acute loss of autoregulatory control [4]. Since, unlike other target organs, the kidney is rigidly embodied in a tight capsule where autoregulation breakdown is, de facto, high hydrostatic pressure [1–2,15]. Malignant hypertension is manifested as acute renal microvascular injury, activation of systemic and intrarenal RAS, histopathological indications of massive vascular endothelial cell destruction and extracellular matrix deposition [16–19]. Thus far, the specific effects of high hydrostatic pressure-induced RAS activation on particular constituents of renal tissue damage have not been investigated systematically. Attempting to approach these issues, we employed the in-vitro model wherein cultured renal epithelial and mesangial, or endothelial cells were exposed to high hydrostatic pressure, so that the potency and duration of the latter would mimic the event of malignant hypertension.

The presence and expression of distinct components of RAS have been well documented in mesangial as well as endothelial and epithelial cells. Adult kidney expresses angiotensin II, AT-1 receptor and, to a much lesser extent, AT-2 receptor proteins in glomeruli, tubular segments and renal vasculature in a normal state [20,21]. A functional prorenin/renin receptor, acting as a cofactor for cleavage of the angiotensinogen molecule to angiotensin I, has been identified on renal mesangium and on vascular smooth muscle cells of cardiac and renal tissue [22]. ACE, a membrane-bound enzyme which cleaves angiotensin I and converts it to angiotensin II, was found not only on the surface of mesangial, but also endothelial and epithelial cells [23]. In addition, a soluble form of ACE also exists, which is released into the body fluids via the proteolytic activity of secretase, another membrane-bound protein [24]. In a diseased kidney RAS expression is activated and the AT-1/AT-2 ratio altered [20,21], indicating the crucial role of RAS activation in the progression of renal disease.

The results hitherto presented ascertain the deleterious role played by renal glomerular mesangium in the pathogenesis of malignant hypertension. In this experimental setting, renal mesangial cells subjected to high hydrostatic pressure have been shown to produce exaggerated amounts of angiotensin II. Apparently, this excessive synthesis of angiotensin II was responsible for augmented proliferation and apoptosis of renal mesangium following pressure application. Indeed, abolishment of enhanced angiotensin II synthesis by captopril treatment, or neutralizing the hormone activity with anti-angiotensin II antibody, or total blockade of angiotensin II receptors by the ‘universal angiotensin II receptor blocker’ saralasine resulted in the complete abrogation of both exaggerated proliferation and apoptosis. In turn, attenuation of both processes by AT-1, but not by AT-2, receptor blockade indicated that, in this experimental set-up, angiotensin II exerted its proliferative and pro-apoptotic effects mainly, but evidently not solely, via the AT-1 receptor. As a possible mechanism, alternative or complementary to the classical AT-1/AT-2 receptor mediated pathway, one might suggest involvement of receptors other than AT-1 or AT-2. Recently, it has been shown that angiotensin II exogenously added to cultures of specific cell lines devoid of AT-1/AT-2, is capable of entering these cells via a pathway(s) unrelated to membrane receptors [25,26]. Once penetrating the cell, angiotensin II proved functional despite the absence of the membrane AT-1/AT-2 receptor activation process [25,26], apparently exerting its specific effects via
putative intracellular binding sites. In this respect, it is also noteworthy that in the present experiment addition of exogenous angiotensin II to mesangial cell cultures prior to pressure enforcement yielded synergistic effects, indicating that 160 mmHg pressure application for 1 h did not completely exhaust the mesangial cell capacity to respond to angiotensin II.

Most importantly, excessive angiotensin II secreted by mesangial cells appeared to be responsible for the damage inflicted on epithelial and endothelial cell populations. Indeed, both epithelial and endothelial cell cultures, while not affected by pressure per se, demonstrated augmented proliferation when exposed either to exogenously added angiotensin II or to mesangial cell medium which, following pressure application, contained sufficient amounts of endogenously produced angiotensin II. Concomitantly, the same angiotensin II induced apoptotic death in epithelial cells, but not in similarly treated endothelial cell cultures. At first glance, it would seem that the latter observation is valid only within the protocol of the present experimental setting. Indeed, as already mentioned, the main components of RAS, such as AT-1, AT-2, prorenin/renin receptors and/or angiotensin II, are expressed in endothelial and in epithelial cells as well as in mesangial cells [27,28]. However, unlike in mesangial cells, no expression of renin, crucial for angiotensin II synthesis, has been found in rat vascular endothelium [29–31]. Moreover, it has been demonstrated that vascular endothelial cells of non-renal origin are incapable of producing angiotensin I and, consequently, angiotensin II, ex vivo if cell isolation is preceded by bilateral nephrectomy [29]. One must thus conclude that in-vivo endothelium is capable of synthesizing angiotensin II only when the catalytic enzyme, i.e. renin, is supplied by an exogenous source. Accordingly, in the present setting, where no exogenously added renin was available in the culture, only the renin-producing mesangial cells were capable of responding to pressure application by augmented angiotensin II formation.

Angiotensin II has been shown previously to stimulate exaggerated apoptosis in a variety of cells [32–35], including endothelial cells, which possess receptors to angiotensin II on their membranes [36,37]. For the HUVEC cell line, the protocols for angiotensin II-induced apoptosis are usually based on at least 18 h incubation [38,39], which was not the case in our experimental set-up. Angiotensin II is also known to stimulate endothelial cells to produce inflammatory chemokines, such as tumour necrosis factor (TNF)-α, matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) [36,40], all of them capable of further potentiating renal tissue damage. Evaluation of inflammatory chemokine formation was beyond the scope of the present study, mainly because a different experimental protocol is needed for such a line of investigation. However, we assessed the content of pro-MMP-1, the precursor of active matrix metalloproteinase-1, in cell culture media following 1 h pressure application. Endothelial cells demonstrated higher basal levels of pro-MMP-1 compared with epithelial or mesangial cultures. This observation was not surprising, since in a normal state MMPs are mainly produced by endothelial cells, macrophages, osteoblasts, etc., rather than by myocyte-like cell types such as mesangial cells [41]. However, in a majority of tissues, in stress-involving situations, enhanced MMP activation plays a significant role in biological processes, culminating in the degradation of collagen and extracellular matrix remodelling [41]. Nevertheless, in all cell cultures employed in the present study, pro-MMP-1 levels were found to be unchanged following 1 h pressure application, whether or not combined with angiotensin II stimulation. This would indicate that in 1 h no appreciable cleavage of the latent, inactive precursor molecule to its active MMP-1 form took place. Previously, in an in-vitro model of transforming growth factor (TGF)-β, TNF-α or phorbol 12-myristate 13-acetate induced inflammation, changes in pro-MMP-1 expression were found only after 24 h, while no effects were detected after 1 h [42]. In a different experimental set-up, employing the application of mechanical stretch to rat tail tendon cells, changes in pro-MMP-1, active MMP-1 or MMP-1 mRNA expression were also detected only after 24 h of tension loading [43]. One would thus conclude that the MMP-1 regulated processes of collagen degradation and extracellular matrix remodelling were not yet set in motion in the acute model of malignant hypertension employed in the present study. Similarly, H2O2 formation was not found to be statistically different from the basal levels in any culture and in any experimental situation, indicating that in this experimental set-up, at this time point, ROS generation did not play an appreciable role in renal cell damage inflicted by enforcement of high hydrostatic pressure.

As already mentioned, there exists a direct communication between mesangial, endothelial and epithelial cells within renal glomeruli [6–8]. Due to this unique structure of a glomerulus, any changes in intracapillary pressure are transmitted directly to the renal mesangium. Mesangial cells respond to the latter by contraction and, consequently, overproduction of angiotensin II, inflammatory chemokines and other autocrine factors, which, in turn, are capable of affecting directly other cell vital functions, such as proliferation and apoptosis.

Mesangial cells originate from myocyte-like cells. They are the only renal cell type capable of contracting in response to pressure enforcement, since they share with myocytes almost all types of intracellular contractile proteins [7,40]. In adult ventricular myocytes, activation of RAS and of angiotensin II synthesis in vivo are
mediated via p53, the cell cycle regulating protein responsible, among other things, for apoptotic cell death. In turn, excessive angiotensin II activates p53 expression in cardiac myocytes, thus creating a positive feedback loop between angiotensin II production and p53 activity [44,45]. Since in renal mesangial cells the possibility of the existence of a similar loop has never been investigated, we assessed p53 expression in mesangial cells, as compared to epithelial and endothelial cells, following application of high hydrostatic pressure. In all three cell types, neither high pressure nor combination of pressure with exogenous angiotensin II resulted in any change of p53 expression. Furthermore, mesangial cells from p53 knockout mice (in which no expression of p53 could be expected) exhibited similar augmentation of angiotensin II synthesis, apoptosis and proliferation following exposure to high hydrostatic pressure. Taken together, these results would indicate that, unlike cardiac myocytes, in this experimental model both exaggerated apoptosis and proliferation are mediated via the angiotensin II pathway independently of p53 activation.

In conclusion, in the in-vitro model of malignant hypertension, renal mesangial cells respond to high hydrostatic pressure by exaggerated angiotensin II synthesis and consequent cell expansion accompanied by enhanced apoptosis. Both processes are, at least in part, carried out via the AT-1 receptor pathway. RAS activation in renal mesangium plays a deleterious role in epithelial and endothelial cell damage. Both epithelial and endothelial cells are unresponsive to high hydrostatic pressure per se. However, apoptosis and/or proliferation can be induced in these cells by angiotensin II, excessively produced by mesangial cells following pressure enforcement. In this model, mesangial cell angiotensin II synthesis, proliferation and apoptosis are carried out via a pathway independent of p53 gene activation.

References


