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Pathogenic Apoptosis of Glomerular Cells in Experimental Nephritis Is Partly Nitric Oxide-Dependent

Zależność patologicznej apoptozy komórek kłębuszków nerkowych
od tlenku azotu w doświadczalnym zapaleniu nerek

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Abstract

Background. Apoptotic mesangial cell death has been regarded mainly as a homeostatic mechanism reducing glomerular hypercellularity in glomerulonephritis. Nonetheless, in the early anti-thymocyte serum nephritis (ATSN) apoptosis has been attributable to excessive generation of nitric oxide.

Objectives. The authors reasoned that NO-dependent mesangial apoptosis may represent a mechanism of pathologic glomerular cell depletion, initiating tissue injury in this nephritis model, along with mesangiolysis mediated by complement.

Material and Methods. In five sets of experiments rats were sacrificed on days 0–4 after injection of ATS and renal tissue samples assessed for glomerular cell count, expression of mRNA for iNOS (RT-PCR *in situ*) and apoptosis of glomerular cells (TUNEL technique). In addition, release of nitrite by isolated renal glomeruli was assessed and glomerular cells checked for proliferating cell nuclear antigen (PCNA) and for infiltrating monocytes/macrophages by immunostaining. In parallel, a group of ATSN rats receiving an iNOS inhibitor, N ω -nitro-L-arginino-methyl ether (L-NAME) was enrolled in each experiment.

Results. On days 0–4 post ATS injection proteinuria gradually increased, whereas in ATSN rats given L-NAME proteinuria was markedly lower (day 4: $p < 0.0005$). Similarly to proteinuria, the extent of mesangiolysis increased on days 0–3 (glomerular cell count: day 0 vs. day 3: $p < 0.005$) and was significantly reduced by L-NAME ($p < 0.005$). Apoptosis of glomerular cells increased during course of ATSN in a manner comparable to that of proteinuria and mesangiolysis and was also partly preventable by L-NAME (apoptotic cell count, day 3: ATS vs. ATS + L-NAME, $p < 0.05$). Treatment with L-NAME did not interfere with glomerular binding of ATS nor deposition of C3 (immunostaining). Apoptotic cell number was significantly inversely correlated with glomerular cell count. Glomerular expression of mRNA for iNOS and generation of NO_2^- in glomeruli peaked on day 2, preceding the highest activity of apoptosis and intensity of glomerulonephritis (proteinuria, mesangiolysis). Generation of nitrite was markedly correlated with the extent of mesangial cell depletion (day 2, $p < 0.04$).

Conclusion. In the mesangiolytic phase of ATSN, high local activity of iNOS is involved in pathogenic apoptosis of mesangial cells (Adv Clin Exp Med 2005, 14, 6, 1181–1189).

Key words: apoptosis, nitric oxide, mesangiolysis, anti-thymocyte serum nephritis.

Streszczenie

Wprowadzenie. Apoptotyczną śmierć komórek mezangialnych uważano głównie za mechanizm homeostaticzny, ograniczający patologiczny rozplęgnięcie komórek w kłębuszkowych zapaleniach nerek (k.z.n.). Tym niemniej, we wczesnych fazach antytymocytarnej k.z.n. apoptozę przypisywano nadmiernemu wytwarzaniu tlenku azotu (NO).

Cel pracy. Autorzy założyli, że zależna od NO apoptoza mezangium może stanowić mechanizm patologicznego zmniejszenia liczby komórek kłębuszka, zapoczątkowującego uszkodzenie tkanki nerkowej w tym modelu doświadczalnym, obok mezangiolyzy wywoływanej przez dopełniacz.

Materiał i metody. W pięciu zestawach doświadczeń szczury uśmiercano w dniach 0–4 po iniekcji surowicy antytymocytarnej (ATS) i we fragmentach tkanki nerkowej badano liczbę komórek kłębuszków, ekspresję mRNA dla indukowanej syntetazy NO (iNOS) metodą RT-PCR *in situ* oraz apoptozę komórek kłębuszków (metoda TUNEL). Oprócz tego oceniano wydzielanie azotynów przez izolowane kłębuszki nerkowe oraz badano ekspresję antygenu jądrowego proliferujących komórek (PCNA) w komórkach kłębuszków, a także obecność naciekających kłębuszki

monocytów/makrofagów (immunoidentyfikacja). W każdym eksperymencie równolegle badano grupę szczurów otrzymujących z wodą do picia inhibitor iNOS, N ω-nitro-L-arginino-metyl eter (L-NAME).

Wyniki. W dniach 0–4 po podaniu ATS białkomocz stopniowo narastał, a u szczurów z ATSN otrzymujących L-NAME białkomocz był znacząco mniejszy (dzień 4: $p < 0,0005$). Podobnie do białkomoczu nasilenie mezangiolizy wzrastało w dniach 0–3 (liczba komórek kłębuszka w dniu 3 była mniejsza niż w dniu 0, $p < 0,005$) i było znacząco zmniejszane przez L-NAME ($p < 0,005$). Apoptoza komórek kłębuszkowych zwiększała się w trakcie ATSN w sposób porównywalny z białkomoczem oraz mezangiolizą i była także częściowo zmniejszana przez L-NAME (liczba komórek apoptotycznych w dniu 3: grupa ATS wobec ATS + L-NAME, $p < 0,05$). Podawanie L-NAME nie miało wpływu na wiązanie ATS ani odkładanie się C3 w kłębuszkach (immunoidentyfikacja). Liczba komórek apoptotycznych była znacząco ujemnie skorelowana z liczbą komórek kłębuszków. Kłębuszkowa ekspresja mRNA dla iNOS i wytwarzanie NO₂ w kłębuszkach były najwyższe w dniu 2. i wyprzedzały najwyższe natężenie apoptozy i nasilenie k.z.n. (białkomocz, mezangioliza). Wytwarzanie azotynów było znacząco skorelowane z nasileniem mezangiolizy (dzień 2., $p < 0,04$).

Wnioski. W mezangiolitycznej fazie ATSN wysoka miejscowa aktywność iNOS jest związana z patologiczną apoptozą komórek mezangialnych (*Adv Clin Exp Med* 2005, 14, 6, 1181–1189).

Słowa kluczowe: apoptoza, tlenek azotu, mezangioliza, zapalenie nerek wywołane przez surowicę antytymocytarną.

Apoptosis, or “programmed” cell death, since its original description by Kerr et al. [1], has been primarily regarded as a homeostatic mechanism, limiting excessive cell proliferation in a wide range of tissues, to restore physiologic balance between destruction and renewal. This significance of apoptosis has been recognized in experimental immune glomerulonephritis characterized by mesangial hypercellularity leading to glomerulosclerosis and obsolescence. Resolution of this entity could be mediated by high apoptotic activity counterbalancing excessive cell division and thus clearing hypercellularity and preventing irreversible fibrosis [2]. Similar observations pertained to human mesangial proliferative nephritis – IgA nephropathy, moreover, results of this study suggested that excessive apoptosis could be harmful, leading to glomerulosclerosis [3]. A link between apoptotic cell death and glomerular lesion has become evident from these and other studies assessing mechanisms of renal cell injury in experimental nephritis [4, 5]. On the other hand, early mesangial cell depletion in the anti-Thy 1.1 nephritis is mediated through cytotoxic nitric oxide (NO) and a blocker of iNOS prevents both mesangiolysis and subsequent glomerular injury [6]. Since NO is capable of triggering apoptosis in mesangial cells [7], the authors hypothesized, that antibody-dependent mesangial cell injury in the initial phase of ATS nephritis may at least in part, be mediated by local generation of nitric oxide causing mesangial apoptosis.

Material and Methods

Experimental Design

In each experiment three groups of rats were enrolled (6 animals in each group): control normal rats, ATS group receiving unmodified drinking water

and ATS rats given drinking solution of L-NAME (this and other reagents used in this study were purchased from Sigma, USA, unless otherwise indicated), at a dose of 60 mg/kg/24 hrs (administration of L-NAME was commenced immediately after injection of ATS). For all animal experimentation described herein the authors adhered to the guidelines for the care and use of laboratory animals established by the local bioethical committee. In a preliminary set of experiments it was demonstrated that administration of L-NAME in drinking water to unmodified healthy rats (60 mg/kg/24 hrs) during 5 days did not modify proteinuria, creatinine clearance or renal histopathology with regard to untreated rats (data not shown). Animals enrolled in the study were sacrificed and experiment terminated on days 0–4 of glomerulonephritis. During experiment rats were housed in metabolic cages on days 0–4 post induction to determine proteinuria in 24 hrs’ collections. Creatinine clearance was calculated from urine creatinine output and serum concentration, using standard formula. Specimens of kidney cortices were fixed in 4% buffered paraformaldehyde and paraffin-embedded to assess histopathology and cell proliferation by staining for PCNA as well as to identify apoptotic figures with the TUNEL method. The extent of mesangiolysis was determined by counting cell nuclei in sections stained with hematoxylin/eosin in 30 glomeruli per section. The presence of infiltrating monocytes/macrophages was examined by immunostaining for the ED-1 antigen and expressed as a number of positive cells per 30 glomeruli. Portions of renal cortices were immediately frozen and kept at –70°C for quantitating expression of iNOS by the *in situ* RT-PCR. Remaining fragments of cortical tissues were utilized for isolation of glomeruli by differential sieving. Secretion of nitrite to supernatants of 48 hrs glomerular cultures was quantitated by the Griess method. Moreover, since admi-

nistration of L-NAME could modulate glomerular ATS binding and subsequent activation of complement, in a separate experiment frozen renal cortical sections were obtained from 4 ATSN control rats and 4 ATSN animals treated with L-NAME, both on day 4 post ATS injection. Sections were thereafter evaluated for glomerular mouse IgG and rat C3 deposits using direct immunofluorescence technique.

Nephritic Model

Male Wistar rats (Institute of Immunology and Experimental Therapy, Wroclaw, Poland) weighing 250 g, fed standard 22% protein diet, were injected in tail vein, under light ether anesthesia, with anti-Thy 1.1 serum (1 ml, kindly provided by R. Johnson, Seattle, USA) recognizing rat mesangial cells. This procedure induces acute glomerular injury with gradual development of proteinuria, mesangiolysis on days 0–3, followed by proliferation of mesangial cells leading to hypercellularity. At termination of experiment, rats were anesthetized with ether, bled through abdominal aorta and kidneys perfused *in situ* with ice-cold saline until bleached, prior to harvest.

Detection of Apoptosis in Sections of Renal Tissue

Apoptotic cells were detected in sections of renal cortices by light microscopic examination ($\times 600$, two independent investigators) of periodic acid-Schiff (PAS)/hematoxylin stained preparations, by identifying characteristic condensed chromatin with perinuclear cytoplasmic halo. Visualisation of apoptotic DNA breaks *in situ* was performed using terminal deoxynucleotidyl transferase (TdT) – mediated dUTP-biotin nick end labeling (TUNEL) method [8] using the *In situ* Cell Detection Kit, POD (Boehringer Mannheim, Germany). Sections (4 μm) were deparaffinized, rehydrated, incubated (15 min, 20°C) with proteinase K and fixed with 4% buffered paraformaldehyde (30 min, 20°C). Next, endogenous peroxidase was quenched and sections incubated (2 min, 4°C) with 0.1% Triton in 0.1% sodium citrate prior to incubation (60 min, 37°C, humid chamber) with solution containing TdT 1 : 100 and biotinylated-dUTP 1 : 200. Finally, sections were exposed to peroxidase labeled anti-fluorescein antibodies (Converter-POD, 30 min, 37°C) and peroxidase substrate DAB (10 min, 20°C). Negative control samples did not include TdT labeling solution, while sections additionally

exposed to DN-ase served as positive controls. At least 50 glomeruli were evaluated per section under light microscope (magnification 500 \times).

Imunohistochemical Evaluation

This method was utilized to determine expression of PCNA antigen and the presence of macrophage ED-1 antigen in glomeruli. After deparaffinating sections and quenching endogenous peroxidase, they were treated with monoclonal mouse anti-PCNA antibody (Dako, Denmark) diluted 1 : 300 (60 min). Goat anti-mouse IgG, peroxidase-labeled (1 : 100, 60 min, Dako) was used as a secondary antibody and DAB (Dako) served as a substrate for peroxidase-catalysed reaction. Negative control consisted of mouse serum instead of anti-PCNA antibody, while for positive control cells from human tonsil were used (Dako). The same method was employed to detect macrophage infiltrates in glomeruli – the primary antibody was monoclonal anti-ED-1 (Serotec, Great Britain). Results were assessed under light microscope. Evaluation of ATS and C3 binding in glomeruli was performed by direct immunofluorescence, using fluorescein isothiocyanate conjugated rabbit anti-mouse IgG (Zymed) and goat anti-rat C3 (Cappel), under fluorescence microscope ($\times 360$ magnification).

Expression of mRNA for the iNOS

A reverse transcriptase polymerase chain reaction (RT-PCR) *in situ* method was utilized, according to description by Bagasra et al. [9]. The iNOS hybridization primer was kindly provided by K.F. Beck (Frankfurt am Main, Germany). 5 μm sections of renal cortices were deparaffinized, heated at 105°C (5 s), fixed in buffered 4% paraformaldehyde and treated with proteinase K (4 $\mu\text{g}/\text{ml}$, Boehringer Mannheim, Germany) for 30 min, at 37°C. RT-PCR reaction was performed using the Super Script One-Step RT-PCR System (Gibco, USA) with primer concentration 0.4 μM . The sequence of the primers was the following: sense: 5'-CCGGATCCTCTTTGCTACTGAGACAGG-3'; antisense: 5'-CCGAATTCGGGATCTGAATGCAATGTT-3'. Twenty cycles were performed at 52°C. Visualization of transcripts was achieved with use of biotin-labeled iNOS DNA primers (Rand D Systems) and alkaline phosphatase-bound streptavidine. The substrate was provided by BCIP/NBT (Dako). Sections of renal cortices of healthy rats served as negative controls. Moreover, RNase-

treated and alkaline-phosphatase-streptavidine only stained cortical sections of ATSN rats were used as additional negative controls. Results were assessed using semiquantitative scale (score 0–6), quantitating intensity of granular stain in glomerular cross-sections.

Secretion of Nitrite by Isolated Renal Glomeruli

In each experiment 5 untreated ATSN rats and 5 ATSN rats obtaining L-NAME were enrolled. Animals were sacrificed on days 1, 2, 3 and 4 after injection of ATS. Healthy naive rats served as controls ($N = 4$). Kidney glomeruli were isolated by differential sieving as described [10], suspended at 500 glomeruli/ml in 25 mM HEPES buffer, pH 7.2, containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 and 5 mM glucose and incubated for 48 hrs at 37°C . NO_2^- concentration was measured in the supernatants of spun mixtures using Griess method [11]. In brief, 250 μl supernatant with 25 μl 12 mM sulfanilamide and 25 μl 6 M HCl was briefly shaken and absorbance read at 548 nm wavelength (reading A_1) in a plate reader (Boehring, Germany). Further on, 25 μl N-(1-naphthyl) ethylene diamine was added and sample left in dark for 20 min followed by another reading (A_2) at the same wavelength. The difference of A_1 and A_2 was extrapolated from standard curve prepared with sodium nitrate dissolved in the above buffer. Results were expressed as pmol of nitrite corrected by protein amount (mg, Bradford method).

Statistical Analysis

Assessment of variance between groups and t-test, corrected for multiple comparisons (Bonferroni) formed basis for analysis of statistical significance. Simple correlations were calculated with Pearson's test. All data are expressed as mean \pm SEM, level of significance for differences set at $p < 0.05$.

Results

Proteinuria and Creatinine Clearance

Urinary protein excretion gradually increased on days 1–4, reaching peak values on day 4. In rats receiving iNOS inhibitor L-NAME, proteinuria was significantly lower on days 2–4 with respect

to control nephritic animals (day 1: ATS 4 ± 4 mg, ATS+L-NAME 5 ± 5 mg; day 2: ATS 34 ± 15 mg, ATS+L-NAME: 12 ± 7 mg; day 3: ATS 33 ± 12 mg, ATS+L-NAME: 16 ± 6 mg; day 4: ATS 55 ± 11 mg, ATS+L-NAME 26 ± 13 mg; $N = 6$, days 2, 4: $p < 0.0005$; day 3 $p < 0.0025$; Fig. 1). Values of creatinine clearance on days 1–4 post induction of nephritis were lower than in healthy animals, although these differences were not statistically significant. Similarly, no considerable differences were found in this regard between days 1–4 in nephritic rats. Administration of L-NAME did not significantly affect results of creatinine clearance during the observation period. These results indicate that inhibition of inducible nitric oxide ameliorates glomerular injury in terms of reducing proteinuria on days 2–4 of the ATSN, without affecting glomerular filtration rate (Fig. 2).

Mesangiolytic

On days 1–3 glomerular cell count markedly decreased with respect to day 0 (healthy control rats), reflecting augmented activity of mesangiolytic (day 0: 40 ± 3 , $N = 6$; day 3: 28 ± 4 , $N = 6$; $p < 0.005$; Fig. 3). Later on (day 4) the number of glomerular cells increased, albeit not significantly. The degree of mesangiolytic was notably decreased in rats receiving L-NAME (cell number, day 3: without L-NAME 28 ± 4 ; $N = 6$; L-NAME: 37 ± 5 , $N = 6$; $p < 0.005$). An experiment assessing kinetics of mesangial cell death within the first 24 hrs of ATSN has demonstrated reduction in cell number already at 2, 6 and 12 hrs post ATS injection (2 hrs: 38 ± 2 ; 6 hrs: 36 ± 4 ; 12 hrs: 35 ± 3), although the differences with initial counts were

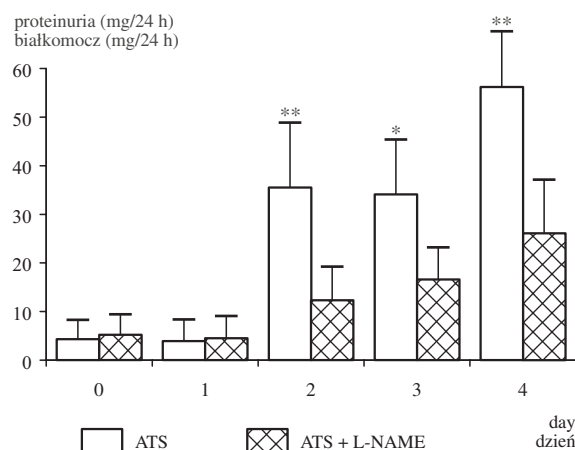


Fig. 1. Proteinuria in rats injected with anti-thymocyte serum (ATS) * $p < 0.0025$, ** $p < 0.0005$: ATS vs. ATS+L-NAME

Ryc. 1. Białkomocz u szczurów po iniekcji surowicy antytymocytarnej (ATS)

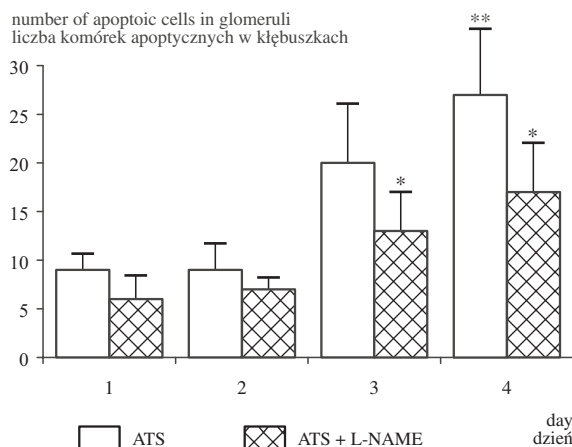


Fig. 2. Mean numbers of apoptotic figures in glomeruli on days 1–4 of the ATSN. (* $p < 0.05$, ** $p < 0.005$)

Ryc. 2. Średnie ilości figur apoptotycznych w kłębuszkach w dniach 1–4 ATSN

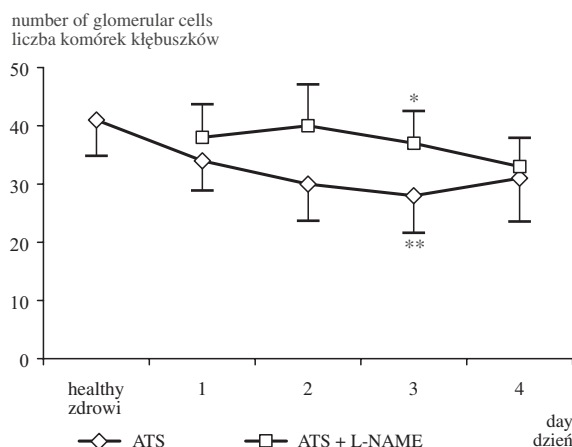


Fig. 3. Mesangiolysis assessed by glomerular cell count on days 1–4 of the ATSN (day 1 vs. day 3: * $p < 0.05$; day 3, ATN vs. ATN+L-NAME: ** $p < 0.005$)

Ryc. 3. Mezangioliza oceniana na podstawie zliczania komórek kłębuszków w dniach 1–4 ATSN

not significant. Respective numbers in rats treated with L-NAME tended to be higher, though not significantly. Thus, similarly to proteinuria, the extent of mesangial cell lysis, as another marker of early glomerular injury in this nephritis model, has been significantly decreased by administration of an iNOS inhibitor on day 3 of ATSN (Fig. 4, 5).

Apoptosis of Glomerular Cells

The intensity of apoptotic cell death (Fig. 6, left panel) clearly augmented on days 3 and 4 when compared to days 1, 2 post nephritis induction, though the difference reached statistical significance on day 4 (day 2: 9.2 ± 3.9 , $N = 6$; day 4:

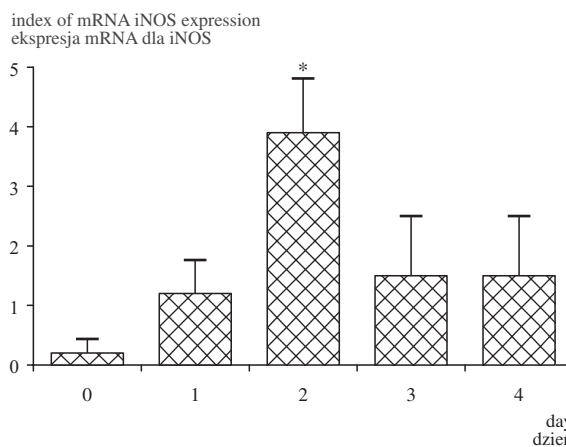


Fig. 4. Expression of mRNA for the iNOS in glomeruli of ATSN rats, visualized with the RT-PCR *in situ* in renal cortical sections and evaluated by a semiquantitative scoring system; day 1 vs. day 2: * $p < 0.0005$

Ryc. 4. Ekspresja mRNA dla iNOS w kłębuszkach nerkowych szczurów z ATSN, uwidoczniła metodą RT-PCR *in situ* i oceniona za pomocą skali półilościowej

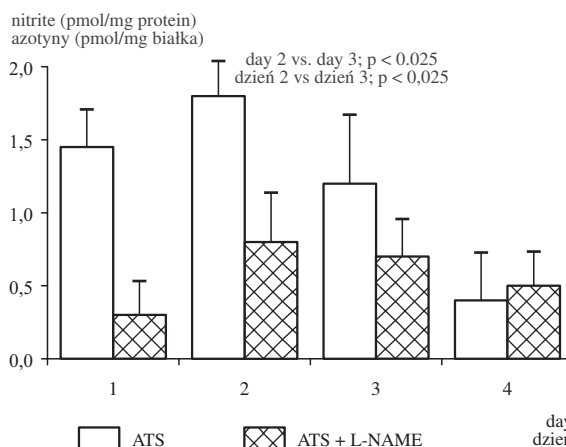


Fig. 5. Generation of nitrite by isolated renal glomeruli by ATSN rats and ATSN rats treated with L-NAME ($N = 5$)

Ryc. 5. Wytwarzanie azotynów przez izolowane kłębuszki nerkowe szczurów z ATSN i szczurów z ATSN leczonych L-NAME

26 ± 5 , $N = 6$; $p < 0.005$; Fig. 2). In rats treated with L-NAME the number of TUNEL-positive cells was markedly lower, than in animals not receiving iNOS inhibitor (days 3 and 4: both $p < 0.05$, $N = 6$). The apoptotic cell number on day 3 was significantly inversely correlated with glomerular cell count ($r = -0.403$; $p < 0.05$), whereas no significant correlation was disclosed with regard to apoptotic activity and proteinuria or creatinine clearance. These data suggest that intensity of glomerular cell apoptosis gradually increases during the first four days of ATSN and is correlated with mesangial cell death in this phase of the disease. Apoptosis is at least in part linked to the activity of nitric oxide, as suggested by its abrogation by L-NAME.

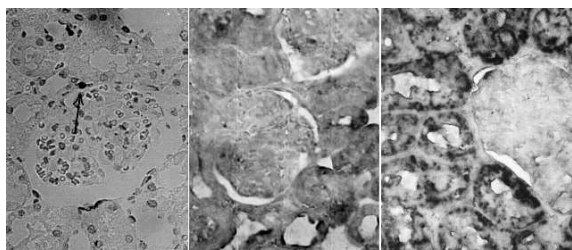


Fig. 6. Renal cortical sections ($\times 500$) of rats with ATSN. Left: Apoptotic figures visualized by the TUNEL method (arrow) in a rat sacrificed on day 4 post induction; middle: iNOS mRNA expression in healthy control rat; right: iNOS mRNA expression (RT-PCR *in situ*) in glomerulus of an ATSN rat on day 2 post induction

Ryc. 6. Preparaty kory nerek szczurów z ATSN. Strona lewa: figury apoptotyczne uwidocznione metodą TUNEL (strzałka) u szczura uśmierzonego w 4. dniu po indukcji; środek: ekspresja iNOS mRNA u szczura zdrowego z grupy kontrolnej; strona prawa: iNOS mRNA w kłębuszku szczura z ATSN w 2. dniu po indukcji

Expression of mRNA for iNOS and Generation of Nitrite in Glomeruli

Whereas preparations of RNase-treated, stained with AP-streptavidine only and healthy control rats' renal cortical sections displayed no appreciable glomerular activity of iNOS mRNA (Fig. 6, middle panel), this activity was demonstrable in glomeruli of ATSN rats (Fig. 6, right panel). As illustrated in Fig. 6, expression of mRNA for iNOS was much higher in cortical tubules than in glomeruli. Semiquantitative assessment has indicated the highest activity of glomerular iNOS mRNA on day 2 (differences with values of days 1, 3 and 4, all $N = 6$, $p < 0.0005$; Fig. 4). On day 0 expression of iNOS mRNA was practically negligible. Assessment of nitrite secretion to supernatants of glomerular cultures was undertaken to differentiate NO_2^- generation in glomeruli from that of tubular cells. Synthesis of nitrite in glomerular cells peaked on day 2 and, on days 1–3, was significantly depressed in rats obtaining L-NAME with regard to nephritic controls not given iNOS inhibitor ($N = 5$, $p < 0.005$; Fig. 5). Glomeruli of healthy control rats released only trace amounts of nitrite (control: 0.02 ± 0.01 pmol/mg protein; control + L-NAME 0.01 ± 0.02 pmol/mg protein, $N = 4$). The release of NO_2^- was significantly correlated with the degree of mesangiolysis (glomerular cell count : concentration of nitrite: $r = -0.302$, $p < 0.04$). Hence, generation of inducible nitric oxide appeared to intensify concurrently with, or immediately prior to a rise of apoptotic activity in glomerular cells at the initial phase of ATSN.

PCNA Antigen and Macrophages in Glomeruli

The presence of proliferating cell nuclear antigen has not been revealed on days 0–3 post nephritis induction, starting to appear on day 4, while ED-1 antigen indicative of monocyte/macrophage infiltration in nephritic glomeruli was identified already on day 1 (3 ± 1 per glomerulus, $N = 6$) and present until day 4 (5 ± 2 , $N = 6$). Number of ED-1 (+) cells was not significantly correlated with any other parameter of the study. These results were not significantly modified by treatment with L-NAME.

Glomerular Binding of Mouse IgG and Rat C3

Granular mesangial pattern of IgG and C3 deposition was visualized in L-NAME-treated and control ATSN rats. Intensity and distribution of deposits was not significantly different in two groups. This findings indicate that in described model L-NAME did not modify binding of ATS antibody and complement in rat glomeruli.

Discussion

Apoptotic figures in mesangial cells during lytic phase of the anti-Thy 1.1 glomerulopathy have been detected already in the early studies of this nephritic model [12]. Although the mechanism of mesangiolysis in ATSN has been attributed to complement activation, some evidence suggests that apoptosis might result from other mechanisms, like interaction of antibodies with mesangial cell epitopes [13]. On the other hand, the activity of l-arginine/nitric oxide pathway has been linked with mesangial cell injury in the anti-Thy 1.1 nephropathy [6, 14] and NO has been found directly cytotoxic to cultured rat mesangial cells [15]. A study of Shimizu et al. [16] has demonstrated complement-dependent mesangial cell destruction in ATSN as a combination of apoptosis and necrosis. Nonetheless, in their experiment, mesangial apoptosis was not exclusively caused by complement activation, since some number of apoptotic figures was still demonstrable in decomplicated animals. Therefore, the authors sought an evidence for a link between generation of nitric oxide and apoptotic death of mesangial cells in ATSN. The results appear to confirm the apoptosis-inducing effect of locally produced nitric oxide in the mesangiolytic phase of anti-Thy 1.1 nephritis. The intensity of programmed death

of mesangial cells, increasing throughout the experiment (days 1–4 of ATSN), significantly correlated with the extent of mesangiolysis. This result implicates apoptosis in pathogenesis of glomerular cell injury in this model of immune nephritis, although a complement-mediated lysis has been thus far regarded solely responsible for mesangial cell depletion in this experimental setting. Nitric oxide-dependent apoptosis may therefore represent yet another mechanism of immune mesangiolysis. Glomerular NO generation peaked prior to the highest expression of mesangial cell apoptosis and administration of an iNOS inhibitor markedly ameliorated both glomerular apoptotic cell death and lysis of mesangial cells. In fact, both apoptosis and mesangial cell lysis was only partly inhibitable by L-NAME, which may indicate that substantial part of cell destruction was nitric oxide-independent and attributable to complement cytotoxicity. Moreover, on days 0–1 notable mesangiolysis was not significantly reduced by iNOS inhibitor, although substantial generation of NO occurs already on day 0 as shown by others [14] and on day 1 (presented results, Fig. 5) of ATSN. Hence, it appears that in the earliest phase (days 0–1) mesangiolysis is mediated by NO-independent mechanism, possibly linked to activation of complement. The complexity of mechanism of mesangial cell killing is further underlined by the fact that on days 2–4 treatment with L-NAME is delaying rather than preventing the decrease in glomerular cell number. Results of presented C3 binding studies indicate that L-NAME did not interfere with complement activation in this phase of ATSN.

On the other hand, the correlation between glomerular apoptosis and proteinuria or filtration rate failed to reach significance, which may indicate that glomerular lesion resulting from apoptotic mesangiolysis was not severe enough to affect glomerular permeability. Relatively low number of infiltrating macrophages in nephritic glomeruli evaluated in presented study, may suggest that mesangial autocrine production in response to local immune/inflammatory stimuli was the major source of apoptosis-inducing nitric oxide. Nonetheless, invading monocytes/macrophages, although scarce in number, may have as well contributed to generation of noxious NO in described model system [17]. In fact, infiltrating mononuclear phagocytes are rather limited in number in the anti-Thy 1.1 nephritis, contrary to other models of immune glomerular injury [18]. The authors are uncertain if high activity of iNOS caused suicidal apoptotic death of host mesangial cells. Nitsch et al. [19] have demonstrated, that apoptosis and expression of iNOS are mutually exclusive in cultured rat

mesangial cells. In described study, apoptotic figures were found within glomerular areas of high iNOS expression, but methods used did not permit to assess if iNOS co-localized with apoptosis in individual cells. Besides, as indicated above, nitric oxide derived from infiltrating macrophages could have also caused apoptosis of mesangial cells. Expression of iNOS mRNA in renal tubulointerstitial cells, although not quantitated in this study, was strikingly high and visibly exceeded that of glomerular cells (Fig. 6). Hence, in order to assess glomerular generation of nitrite and separate it from tubules-derived NO, the authors isolated glomeruli from renal cortices by differential sieving.

Absence of glomerular cell proliferation up to day 4 of ATSN, judged from results of immunostaining for PCNA, ruled out a possibility that mesangial apoptosis might have represented a compensatory homeostatic mechanism, eliminating excessive cells from glomeruli. Accordingly, NO-dependent apoptosis in the early, mesangiolytic phase of ATSN emerges as a pathogenic mechanism, contributing to development of glomerular injury initiated by anti-mesangial cell antibody. Although the lesion induced by glomerular apoptosis was not particularly severe, failing to correlate with proteinuria and creatinine clearance, it may have affected the later course of the disease. Peters et al. [14] have demonstrated that enhanced iNOS activity at the onset of ATSN is linked with profound mesangiolysis at the early phase of the disease and subsequently results in accentuated glomerular fibrosis from day 6 post induction. In presented study, the course of disease has not been followed beyond day 4, though it is conceivable that increased mesangiolysis due to NO-dependent apoptosis might have caused a comparable degree of fibrosis in the later phase of ATSN.

A relation between excessive apoptosis of proliferating mesangial cells and subsequent development of glomerulosclerosis has been observed in human IgA nephropathy [3]. Aberrant apoptosis of glomerular endothelial cells affecting physiologic capillary remodelling has been implicated in sclerosis and obsolescence of renal tufts in proliferative phase of anti-Thy 1.1 nephropathy [20]. Similarly, a correlation has been observed between iNOS-related apoptosis and glomerulosclerosis in human lupus nephritis [21]. Indeed, the activity of oxygen radicals and nitric oxide have been implicated in apoptosis of glomerular cells [7, 22]. Mechanisms of NO-dependent mesangiolysis in ATSN may include impairment of mesangial cell adhesion to extracellular matrix, followed by detachment and death [23]. Interestingly, Floege et al. [24] have demonstrated that administration of antibodies neutralizing fibroblast growth factor-2

(FGF-2) reduces early mesangial cell injury in ATSN with a decrease of glomerular production of nitric oxide. These data may indicate a link between FGF-2 activity and NO-mediated mesangiolysis.

Presented results, as well as those of other authors [6, 14] indicate a pathogenic role of nitric oxide in ATSN, whereas in other models of acute glomerular injury it appears to exert an opposite, renoprotective effect [25]. Hence, it is unclear, which factors determine the influence of NO on development and course of glomerulonephritis: certainly its local concentration, activity of oxygen radicals and endogenous NO-antagonists must

be considered in this regard. Differences in immunopathogenesis of various models of acute glomerulonephritis may affect relations between the above determinants of l-arginine/nitric oxide pathway resulting in either nephritogenic or renoprotective influence of iNOS expression. The latter appears to prevail in chronic models of glomerulopathies where beneficial effect of nitric oxide on glomerular hemodynamics is important [26]. Presented data indicating that NO-dependent apoptosis mediates mesangiolysis in the early ATSN enlarge body of evidence speaking in favor of nephritogenic potential of l-arginine/nitric oxide pathway in development of acute glomerular injury.

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