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The Pathogenetic Role of Reactive Oxygen Species in Aminonucleoside Nephrosis†

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= Abstract = **We studied the pathogenetic role of reactive oxygen species (ROS) in rats with puromycin aminonucleoside nephrosis (PAN). Heavy albuminuria with markedly decreased density of the anionic sites (AS) on glomerular basement membrane (GBM) (2. 6 ± 0. 98 compared to 20. 0 ± 1. 61 AS/1,000nm GBM in control) developed 7 days after PA injection. The malondialdehyde (MDA) levels in kidney homogenates increased gradually (1. 16 ± 0. 18 at day −1 to 1. 97 ± 0. 23/g protein at day 5). While catalase or dimethyl sulfoxide, administered with PA, did not affect the course of PAN, superoxide dismutase and allopurinol reduced proteinuria and decreased loss of the AS (11. 7 ± 2. 80 and 13. 7 ± 1. 27 AS/1,000nm GBM, respectively) at day 7. These findings suggest that proteinuria in PAN results from the loss of GBM AS, in which ROS generated by xanthine oxidase system plays an important role.**

Key Words: *Puromycin aminonucleoside nephrosis, Anionic site, Reactive oxygen species, Xanthine oxidase, Malondialdehyde*

INTRODUCTION

Massive proteinuria, the primary abnormality in nephrotic syndrome, results from the altered permselectivity of the glomerular capillary wall. The permselectivity depends on the size and charge of macromolecules including albumin (Chang *et al.* 1975; Deen and Satvat 1981). To date, the anionic sites (AS), which are mostly heparan sulfate proteoglycans(Kanwar *et al.* 1984), on glomerular basement membrane (GBM) are believed to be the main charge-selective barrier for which there is much experimental evidence (Kanwar and Farquhar 1979).

Single i. v. injection of 6-methyl-amino- 9-(3'-amino-3'-deoxyribosyl) purine (puromycin aminonucleoside, PA) in a rat induces nephrotic syndrome (PA nephrosis, PAN) (Frenkel *et al.* 1955). Several experiments have revealed the defective charge-selective barrier function of GBM with markedly reduced AS in PAN rats (Olson *et al.* 1981). Although the exact pathogenesis of PAN is not yet clear, there have been a series of papers (Thakur *et al.* 1988; Beaman *et al.* 1987) supporting the view that oxidative injury due to increased generation of reactive oxygen species (ROS) plays a significant role in PAN since the first report by Diamond *et al.*

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(1986). However, they only presented indirect evidence like the antiproteinuric effects of several ROS scavengers or enzyme inhibitors administered with PA.

In order to clarify the role of ROS in PAN further, we measured malondialdehyde (MDA) levels in the kidneys as a marker for lipid peroxidation in addition to the effects of several intervening drugs. And the effects of those drugs on the changes of GBM AS were also studied.

Materials and Methods

Induction of PAN

Male Wistar rats weighing around 200g were used. On day 0, both femoral veins were cannulated, and a single dose of PA (5mg/100g BWt) was injected via one side of the femoral vein cannula over 5 min. The other side of the cannula was used for injecting the intervening drugs. PA and other drugs for injection were dissolved in 3ml of 0.9% NaCl. For control rats, the same volume of 0.9% NaCl was injected.

Measurement of malondialdehyde (MDA) levels in kidneys

Among a group of rats, five animals each were sacrificed on day -1, day 1, day 3 and day 5 after PA injection. And the MDA concentration in the kidney homogenates was measured by thiobarbituric acid method (Bidlack and Tappel 1973) to estimate the degree of lipid peroxidation. The absorbance measured at 533nm with a spectrophotometer for MDA was corrected for a unit of protein (absorbance/gm protein).

Modification of PAN with the intervening drugs

Animals were grouped into 6 groups (Group A to F). Five (Group A to E) of them were PAN groups and one (Group F) was a control. Superoxide dismutase (SOD, 1.5mg/100g BWt), catalase (CATA, 4mg/100g BWt) and dimethyl sulfoxide (DMSO, 8mg/100g BWt) were infused over 30 min prior to and 30 min following PA injection in Group B, C, and D, respectively. In Group E, 10mg/100g BWt of allopurinol (ALLO) was administered orally every 12 hours 5 times prior to and another 5 times after PA injection. All the drugs and PA were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Biochemical study

Two samples of 24 hour urine were collected by housing individual rats in metabolic cages at day 0 and day 7. At the day of sacrifice (day 7), open cardiac punctures for blood sampling and prompt removal of both kidneys were done under ether anesthesia. Albumin and creatinine were measured with both urine and blood samples, and cholesterol was checked additionally in blood samples.

Pathologic study

Some parts of cortices separated from the kidneys were processed by a routine method for light microscopic examination with hematoxylin-eosin staining. Other parts of cortices were cut into small cubes of 0.5mm X 0.5mm X 0.5mm in size. The cubes were stained with PEI (MWt 1,800, Polyscience, Warrington, PA, USA) in vitro according to the procedure described elsewhere (Okada *et al.* 1986). Then a routine procedure for electron microscope like gradual dehydration with ethanol and embedding in POLY BED 812 (Polyscience, Warrington, PA, USA) followed. Thin sections (60-80nm) were stained with 4% aqueous uranyl acetate and lead acetate, and examined with a Hitach H-600 electron microscope. The numbers of AS stained as electron-dense dots along lamina rara externa (LRE) were measured at X 20,000 magnification. The actual length of GBM checked for the morphometry was 20 — 40 nm for each specimen, and areas with double layers of AS in LRE indicating oblique sections were excluded for measurement.

Statistical Analysis

Both biochemical and MDA measurements

were duplicated. The mean value in a group was described with ± 1 SE. Comparisons between group data were performed by the unpaired or paired Student t-test, and $p<0.05$ was considered statistically significant.

RESULTS

MDA levels in kidney tissue

The MDA level in the kidneys prior to PA treatment was 1. 16 ± 0. 18/g protein. The levels increased gradually following PA treatment, e. g. 1. 29 ± 0. 18/g protein on day 1, 1. 62 ± 0. 19/g protein on day 3, and 1. 97 ± 0. 23/g protein on day 5(Fig. 1).

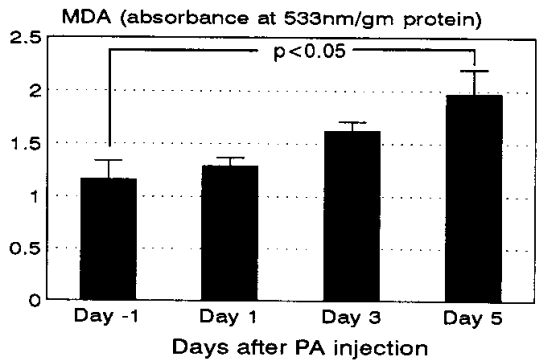

Fig. 1. The MDA Levels serially measured in the kidneys after PA administration. The levels increased gradually following PA treatment from 1.16 ± 0.18/g protein on day -1 to 1.29 ± 0.18/g protein on day 1, 1.62 ± 0.19/g protein on day 3, and 97 ± 0.23/g protein on day 5.

While urinary creatinine excretion was not changed significantly (data not shown), albumin excretion showed variable increases in all PA treated groups (Groups A – E) at day 7. It

Biochemical studies

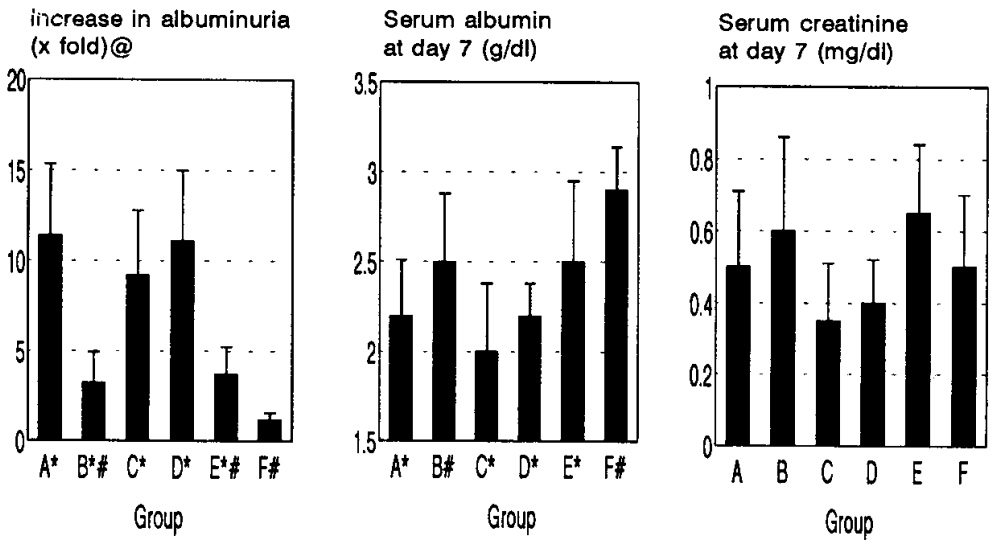

Fig. 2. The biochemical data from each group on day 7. Significant albuminuria and hypoalbuminemia developed in Group A animals at day 7 compared to the control Group F. The degree of albuminuria and hypoalbuminemia were significantly, although not completely, inhibited in Group B and E, but not in Group C and D. The serum creatinine levels were not different among groups(* significantly different from Group F($p < 0.05$), # significantly different from Group A($p < 0.05$), @ratio of the amount of urinary albumin excretion at day 7(mg/day/g BWt) to that at day 0).

increased about 11 fold in Group A. The increments were significantly, although not completely, inhibited in Group B and E, but not in Group C and D. Group A – E developed significant hypoalbuminemia and hypercholesterolemia (data not shown) at day 7 compared to Group F. And the changes in both parameters were partly but significantly inhibited in Group B. The serum creatinine levels were not different among groups(Fig. 2).

Pathologic studies

Light microscopic examinations of the kidneys revealed no significant change in all PA treated groups (Group A – E) except for focal proteineous materials in tubular lumens and/or Bowman spaces. The main pathologic changes were noted, with electron microscopic examinations, in epithelial cells like focal detachment from GBM or effacement of foot processes, microvillous changes and attenuation of cytoplasms. Again, the changes were less prominent in Group B and E. The AS stained by in vitro PEI were observed as regularly arrayed electron-dense dots along LRE and LRI. The densities of the AS in LRE were measured as 20.0 ± 1.61/1,000nm GBM in Group F, and decreased to 2.6 ± 0.98/1,000nm GBM in Group A. The decrements in the densities in Group B and E, again, were partly inhibited, e. g. 11.7 ± 2.80 and 13.7 ± 1.27/1,000nm GBM, respectively(Fig. 3 and 4).

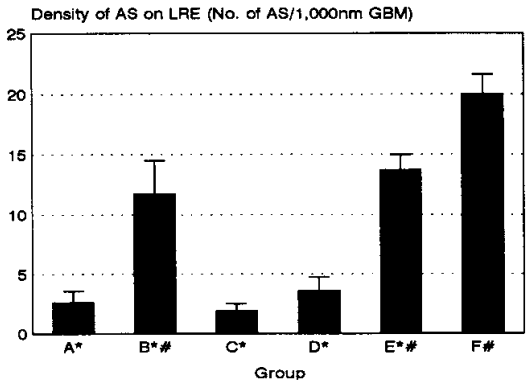

Fig. 3. The density of AS on LRE in each group at day 7. The density was measured as 20.0 ± 1.61/1,000nm GBM in the control Group F, and decreased to 2.6 ± 0.98 /1,000nm GBM in Group A. The decrements in the densities in Group B and E were partly inhibited, but not in Group C and D.(* significantly different from group F($p < 0.05$), # significantly different from Group A($p < 0.05$))

DISCUSSION

Free radicals or reactive oxygen molecules generated during metabolic processes, either normal or abnormal, of cells are collectively called ROS, and are known to be able to cause various cell and tissue injuries including many experimental and clinical renal diseases (Diamond 1992). In 1986, Diamond *et al.* observed that SOD (a superoxide anion scavenger) and ALLO (a xanthine oxidase inhibitor) had some protective effect for proteinuria in PAN rats. And because hypoxanthine, an intermediate metabolite of PA, can act as substrate for ROS generation in the cellular system via the intracellular xanthine oxidase enzyme system (Parks and Granger 1983), they hypothesized that the glomerular lesion in PAN was mediated by ROS. And the lack of protective effect with CATA (a hydrogen peroxide scavenger) or DMSO (a hydroxyl radical scavenger), as in this study, supports the role of superoxide anion as a major pathogenic ROS. However, in other reports, hydrogen peroxide or hydroxyl radical was also found to play some role (Thakur *et al.* 1988; Beaman *et al.* 1987). Beaman *et al.* (1987) found that both SOD and polyethylene glycol-coupled CATA (PEG-CATA) diminished proteinuria in PAN rats, which supported the pathogenetic roles of not only superoxide anion but also hydrogen peroxide. Thakur *et al.* (1988) evaluated the effect of dimethyl thiourea, sodium benzoate (another hydroxyl radical scavengers) and desferoxamine (an iron chelator) in PAN rats. And the antiproteinuric effects of all these drugs implicated the patho-

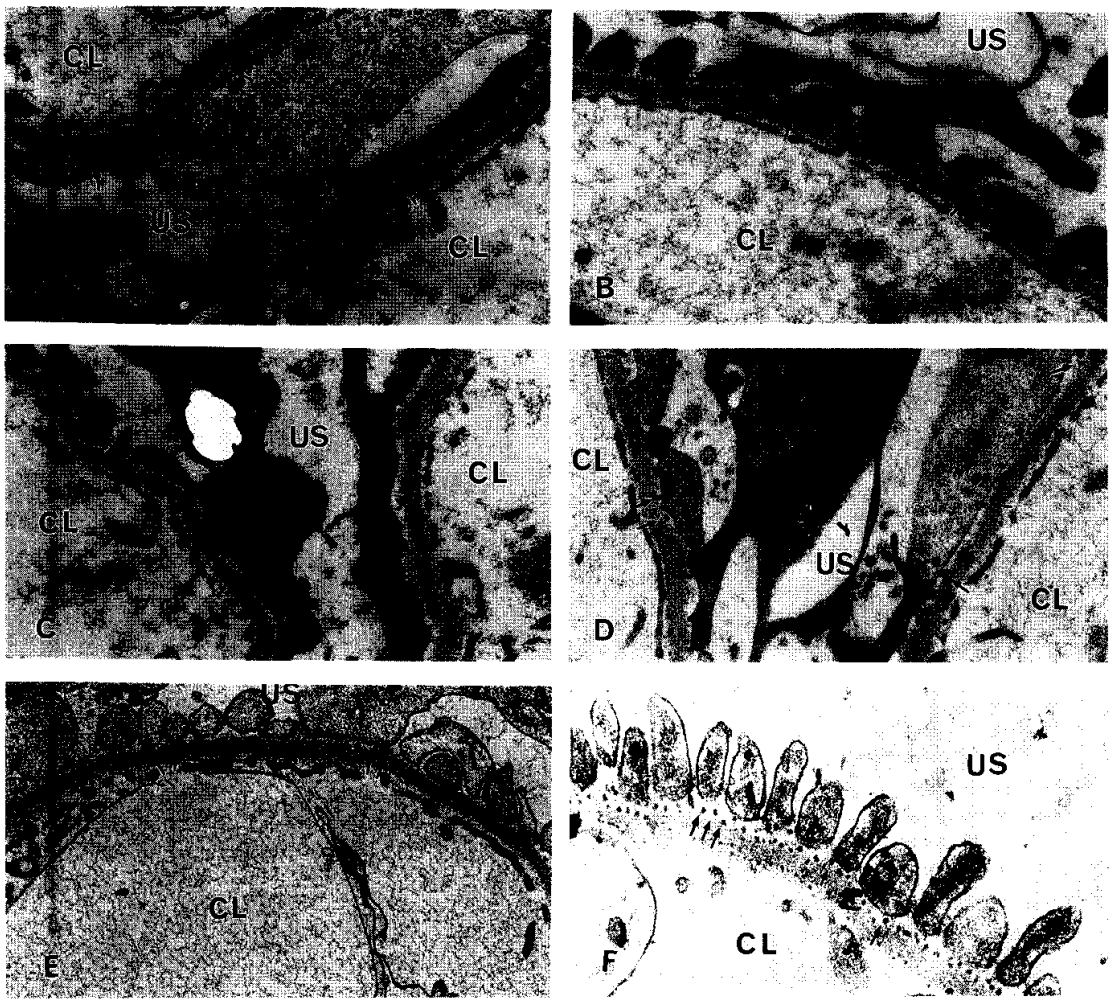

Fig. 4. The electron microscopic findings of glomeruli with in vitro polyethyleneimine staining of anionic sites (X20,000). Panels A—F are Group A—F, respectively. Marked reduction of anionic sites (arrows) along both sides of glomerular basement membranes in number and diffuse effacement of foot processes are noted in Panel A, C, and D compared to Panel F. The findings in Panel B and E are intermediate in degree. (CL, capillary lumen ; US, urinary space)

genetic role of the hydroxyl radical. The different results among these studies might result from the differences in pharmacokinetic characteristics or the indirect secondary actions of the drugs used. CATA has a very short (3 — 5 minutes) circulatory half life and a large molecular weight (242,000) (Auchowski *et al.* 1977). So one can not be sure how much exogenously administered CATA traverses GBM, gets into the cytoplasms of glomerular cells and acts as a scavenger. On the other hand, conjugating PEG to CATA can increase the half life over 24 hours (Auchowski *et al.* 1977) provide act more consistent action. DMSO can generate toxic methyl radicals or methylperoxy radicals secondarily by interacting with hydroxyl

radical, and this secondary toxic effect may offset the primary protective scavenging effect (Raleigh and Kramer 1981). And there has been a report that DMSO potentiated PAN (Spear *et al.* 1987). Other hydroxyl radical scavengers without this secondary action can show protective effects.

In general, there are 3 requisites to confirm the pathogenetic role of ROS in a particular disease process (Baud *et al.* 1992). First, local generation of ROS and its increment along the disease course should be confirmed. Second, experimentally generated ROS via chemical or enzymatic reactions can produce similar manifestations. And third, specific scavengers or inhibitors for corresponding ROS molecule(s) or enzyme(s) can prevent or reduce the injury. However, in previous papers (Thakur *et al.* 1988; Beaman *et al.* 1987; Diamond *et al.* 1986), only the effects of several scavengers and/or enzyme inhibitors were measured. We didn't measure individual ROS in this study, either. Instead, we checked MDA levels. In the presence of ROS, cellular signals of lipid peroxidation by ROS should be present. MDA is an end product of lipid peroxidation. So, increased levels of MDA meant increased generation of ROS in PAN kidneys.

We also studied the GBM AS by electron microscopic morphometry to correlate their changes with the effects of the intervening drugs. What is the meaning of loss of GBM AS in PAN? This morphologic change begins to be observed as early as 24 hours following PA treatment and precedes development of proteinuria, which suggests that it is not the result but the cause of proteinuria (Mahan *et al.* 1986; Washizawa *et al.* 1989). Captopril, an angiotensin converting enzyme inhibitor, is well known to have some antiproteinuric effect in various experimental and clinical renal diseases including PAN (Fogo *et al.* 1988). This effect is at least partly independent of its antihypertensive ability (Fogo *et al.* 1988) and may result from a sulfhydryl group in its molecule (Jaffe 1986), which can act as a scavenger of ROS (Westlin and Mullane 1988). In PAN, the antiproteinuric effect of captopril correlates with its protective effect on the loss of AS (Azuma *et al.* 1990). However captopril has no effect on an adriamycin nephrosis model (Bertani *et al.* 1982; Beukers *et al.* 1988; Weening and Rennke 1983), which is another rat model of massive proteinuria. In this model, the major defect is on size-selective barrier function of GBM without affecting charge-selective barrier function and GBM AS (Weening and Rennke 1983). These findings support the view that the effect of captopril by scavenging ROS may be protection of GBM AS.

So we can speculate the pathogenesis of PAN as follows; PA increases ROS generation in kidney tissue via intracellular xanthine oxidase enzyme system, which, in turn, results in loss of GBM AS by either directly damaging GBM or altering metabolism of glomerular cells (especially epithelial cells). And then the defective charge-selective barrier function of GBM, massive proteinuria, and nephrotic syndrome follow.

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