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TREATMENT WITH 1, 25-DIHYDROXYVITAMIN D3 PRESERVES GLOMERULAR SLIT DIAPHRAGM-ASSOCIATED PROTEIN EXPRESSION IN EXPERIMENTAL GLOMERULONEPHRITIS

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In this study, we investigated the effect of $1,25(OH)_2D_3$ on proteinuria and on the alteration of slit diaphragm-associated proteins induced by anti-Thy 1.1 in Wistar rats. Four groups of animals were studied: group I, anti-Thy 1.1 treated rats; group II, anti-Thy1.1 treated group that at day 2, after the onset of overt proteinuria, started the treatment with $1,25(OH)_2D_3$; group III, normal control rats injected with vehicle alone; group IV, rats that received only $1,25(OH)_2D_3$. At day 2, in group I and II, before the administration of $1,25(OH)_2D_3$, protein excretion was significantly increased when compared to controls. Overt proteinuria was maintained until day 14 in group I whereas in group II protein excretion was significantly reduced from day 3 to day 14. Moreover, treatment with $1,25(OH)_2D_3$ abrogated podocytes injury, detected as desmin expression and loss of nephrin and zonula occludens-1 (ZO-1), two slit diaphragm-associated proteins, and glomerular polyanion staining, that were observed in group I. In conclusion, these results suggest that $1,25(OH)_2D_3$ administrated with a therapeutic regiment may revert proteinuria, counteracting glomerular podocyte injury.

Several studies indicated that 1,25-Dihydroxyvitamin D_3 ($1,25-(OH)_2D_3$) may affect cell proliferation and differentiation (1-2), and immune response (3-4).

Anti-proteinuric and anti-inflammatory effects of $1,25-(OH)_2D_3$ have been shown in different experimental models of glomerular injury including Heymann nephritis (5), mercuric chloride-induced autoimmune disease in BN rats (6), and spontaneous lupus in MRL/L mice (7). Moreover, we demonstrated that $1,25-(OH)_2D_3$ administration reduced proteinuria, as well as recruitment of inflammatory cells and mesangial proliferation, in the anti-Thy-1.1-induced glomerulonephritis (GN) in rats (8). $1,25-(OH)_2D_3$ was also shown to reduce proteinuria, cell proliferation, glomerular growth and glomerulosclerosis in a model of subtotal nephrectomy (9). Therefore, $1,25-(OH)_2D_3$ seems to inhibit proteinuria in different experimental models independently from the initial pathogenic mechanism. These observations prompt us to investigate the mechanism of anti-proteinuric effect of $1,25-(OH)_2D_3$.

Recent studies focused on a central role of the podocyte slit diaphragm in maintaining the glomerular permeability (10). In particular, the functional role of slit diaphragm-associated proteins such as nephrin, podocin, CD2-associated protein, alpha-actin 4 and neph1 in maintaining the size-selective barrier has been defined (11). Besides

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inherited nephrotic syndromes, a correlation between changes in nephrin expression and proteinuria has been shown in several experimental models of GN (12-17). We and others have recently shown a reduced expression of nephrin also in patients with diabetic nephropathy (18-19) and primary acquired nephrotic syndromes, such as membranous GN, minimal change GN, and focal segmental glomerulosclerosis (19, 20-23).

The aims of this study were to investigate: (i) whether treatment with 1,25-$(OH)_2D_3$ may reduce proteinuria when started after the induction of glomerular injury; (ii) whether it may protect glomerular podocytes, and consequently, the expression of nephrin and zonula occludens-1 (ZO-1), two slit diaphragm-associated proteins.

MATERIALS AND METHODS

Experimental design

Seventy-two eight-week-old female Wistar rats (Harlan Italia, Udine, Italy) were housed in single metabolic cages at constant room temperature (20°C) and humidity (75%) under a controlled light/dark cycle. Rats were fed a standard chow diet and they had free access to drinking water. All experiments were performed in accordance to the guidelines of our local ethics committee. Rats were randomized to one of the four following groups of treatment (18 rats/group):

1. Rats with mesangial proliferative glomerulonephritis (MPGN rats). At day 0, experimental mesangial proliferative glomerulonephritis was induced in female Wistar rats by intravenous injection of 100 μL of ascites fluid containing the mouse IgG anti-Thy-1.1 monoclonal antibody (Cederlane, Ontario, Canada), as originally described by Bagchus et al. (24).
2. Rats with mesangial proliferative glomerulonephritis treated with 1,25-$(OH)_2D_3$ (MPGN rats tretaed with 1,25-$(OH)_2D_3$). At day 0, rats received mAb anti-Thy- 1.1, as in group I. From day 2 to the end of experiment rats were treated with 1,25-$(OH)_2D_3$ (Calcijex; Abbott, North Chicago, IL, USA) at the dose of 25 ng/100 g body wt/day.
3. Normal control group. At day 0, rats were injected i.v. with 100 μL of ascitic fluid without mAb anti-Thy-1.1 (vehicle).
4. Control group treated with 1,25-$(OH)_2D_3$. At day 0, rats were injected i.v. with 100 μL of ascitic fluid without mAb anti-Thy-1.1. From day 2 to the end of the experiment rats were treated with 1,25-$(OH)_2D_3$ at the dose of 25 ng/100g body wt/day, as previously reported (8). MAb anti-Thy1.1 and vehicle were given by intrafemoral venous injection, whereas 1,25-$(OH)_2D_3$ (Calcijex, Abbott Laboratories, Abbott Park, Illinois, USA) was administered subcutaneously. After the dose administration, serum and kidney levels of calcitriol peaked at hour 2 and they were also detectable at hour 24, as previously reported (25-26). The study was stopped at day 14.

Measurements of proteinuria

Twenty four hour urine samples were collected. Proteinuria was measured daily by using Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, München, Germany) and bovine serum albumin (Sigma Chemicals Co., St. Louis, MO, USA) as standard.

Morphologic studies

At day 4, 7, and 14, six rats from each group were sacrificed by an anesthetic overdose, and the kidneys were removed. One part was fixed in 2.5 % paraformaldehyde (PFA) for 24 hours, suspended in 0.15 mol/L NaCl containing 30% sucrose for 24 hours, snap-frozen in liquid nitrogen cooled with isopentane, and stored at -70°C. Another part was fixed in 10% neutral-buffered formalin and embedded in paraffin. Hematoxylin and eosin (HE) and periodic acid-Schiff stainings were performed to investigate glomerular morphology.

Immunofluorescence studies

Three-μm-thick cryostat kidney sections were dried for 30 minutes at room temperature, post-fixed in 3.5% PFA for 20 minutes and washed with phosphate-buffered saline (PBS). The sections were then incubated with rabbit anti-rat nephrin (1:640) (NPHN11-A, Alpha Diagnostic, San Antonio, TX) (23) or with rabbit anti-ZO-1 (Santa Cruz Biotechnology Inc., Santa Cruz, California, U.S.A.) overnight at 4°C, washed in PBS, and incubated with FITC-conjugated anti-rabbit IgG for 1 hour at room temperature. Nephrin and ZO-1 expression were analyzed semi quantitatively by measuring fluorescence intensity using a digital image analysis software (Windows Microimage 3.4, CASTI Imaging, Venezia, Italy) on images captured with a low light video camera (Leica DC100, Wetzlar, Germany) with a 180 μm diameter field. The results were expressed as relative fluorescence intensity on a scale from 0 (fluorescence of background of tissue) to 255 (fluorescence of standard filter).Control experiments included incubation of sections with nonimmune isotypic control antibodies or the omission of the primary antibodies followed by the appropriate labeled secondary antibodies.

Immunohistochemical staining

Immunohistochemical staining was performed as previously described (8). Briefly, 3-μm-thick paraffin-embedded kidney sections were boiled in citrate buffer (0.01

mol/L, pH 6.0) in a microwave oven (2x10 minutes, 600 W) for antigen retrieval. Endogenous peroxidase activity was quenched with 3% hydrogen peroxidase for 10 minutes. Blocking was achieved by incubating the samples in a solution of 10% bovine serum albumin and of 1.5% aspecific antiserum in PBS for 1 hour at room temperature. The sections were then incubated with primary antibodies overnight at 4°C. Proliferating cells, monocytes and desmin positive podocytes were marked with a biotinylated mouse anti-proliferating cell nuclear antigen (PCNA, Zymed, South San Francisco, CA, USA), anti-ED1 mAb (MCA341R, Serotec Ltd, Oxford, U.K.) and anti-desmin mAb (Sigma Chemicals Co., St. Louis, MO, USA), respectively. Anti-PCNA treated sections were incubated with streptavidin-peroxidase for 10 minutes, and the reaction was detected with peroxidase substrate containing diaminobenzidine chromogen (DAB). Anti-ED1 and anti-desmin treated sections were incubated with a streptavidin-peroxidase conjugate anti-mouse secondary antibody for 30 minutes at room temperature and then with a substrate-chromogen mixture containing 3-amino-9-ethyl-carbazole (AEC) for 10 minutes at room temperature. Finally, all sections were counterstained with hematoxylin. For the negative controls, the specific antibodies were omitted. To evaluate glomerular apoptosis, TUNEL assay (*In situ* cell death detection kit-POD, Roche Diagnostics S.p.A., Italy) was performed on formalin fixed-paraffin embedded kidney tissue, as previously described for PCNA. Terminal deoxynucleotidyl transferase (TdT) was omitted from the nucleotide mixture as negative control. Kidney sections treated with DNAse were used as positive controls. Calculation of the number of positive cells per glomerular cross section was performed by three different investigators in a blinded fashion using coded samples.

Staining of glomerular polyanions

The presence of glomerular polyanions (GPA) was evaluated by the colloidal iron as previously reported (27). Briefly, 4-μm-thick paraffin-embedded kidney sections were rinsed in 12% glacial acetic acid aqueous solution, and stained in the colloidal iron solution for 1 hour and 30 minutes. Sections were finally rinsed four times in the acetic acid solution. The pH of both staining and rinsing solutions was between 1.1 and 1.3 to ensure selective staining of acidic polysaccharides. The Prussian blue color was developed in a freshly prepared mixture of equal parts of 2% hydrochloric acid and 2% potassium ferrocyanide.

Statistical analysis

ANOVA followed by Neumman-Keuls', Dunnet's or Tukey's multicomparison tests were used where appropriated. Results were expressed as mean value ± standard deviation; the null hypothesis was rejected when $p<0.05$.

RESULTS

As depicted in Figure 1, at day 2, proteinuria was significantly increased in MPGN rats and overt

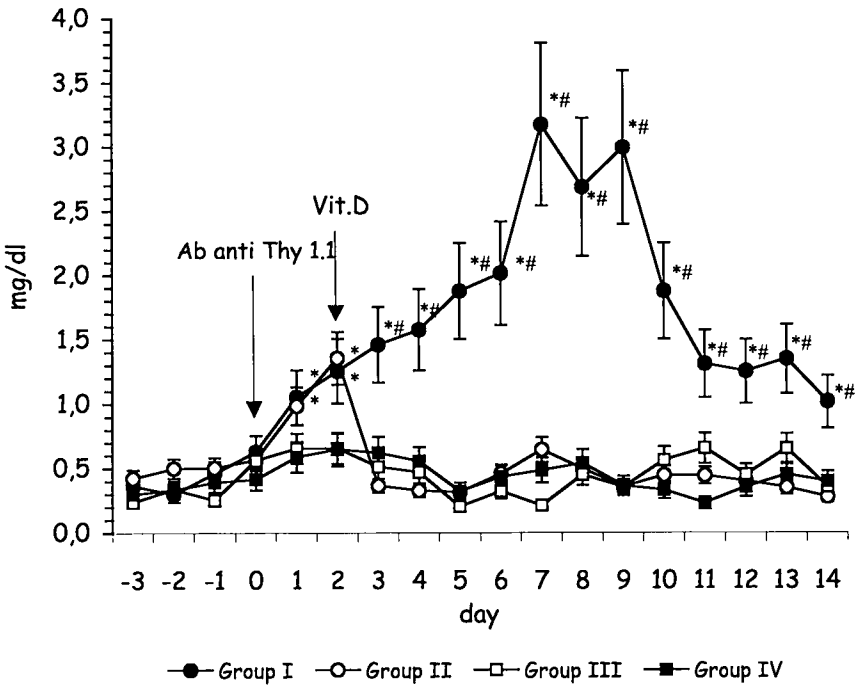


Fig. 1. *Twenty-four hour protein excretion in the experimental groups. Proteinuria was measured daily starting 3 days before the administration of anti-Thy 1.1 antibody up to day 14. group I includes 18 rats injected with 100 μL of ascite fluid containing mouse monoclonal anti-Thy 1.1 antibody. Groups II includes 18 rats injected with anti-Thy 1.1 antibody and 2 days after the induction of Thy 1.1 glomerulonephritis treated with 1,25-$(OH)_2D_3$ (25 ng/100 g body wt/day). Controls include 18 rats/group injected with 100 μL of non-immune ascite fluid alone (group III) or followed by treatment with 1,25- $(OH)_2D_3$ (25 ng/100 g body wt/day) (group IV). ANOVA with Newman-Keul's multicomparison test was performed among group I vs. group III and IV (* P < 0.01), and group I vs. groups II (# P < 0.01).*

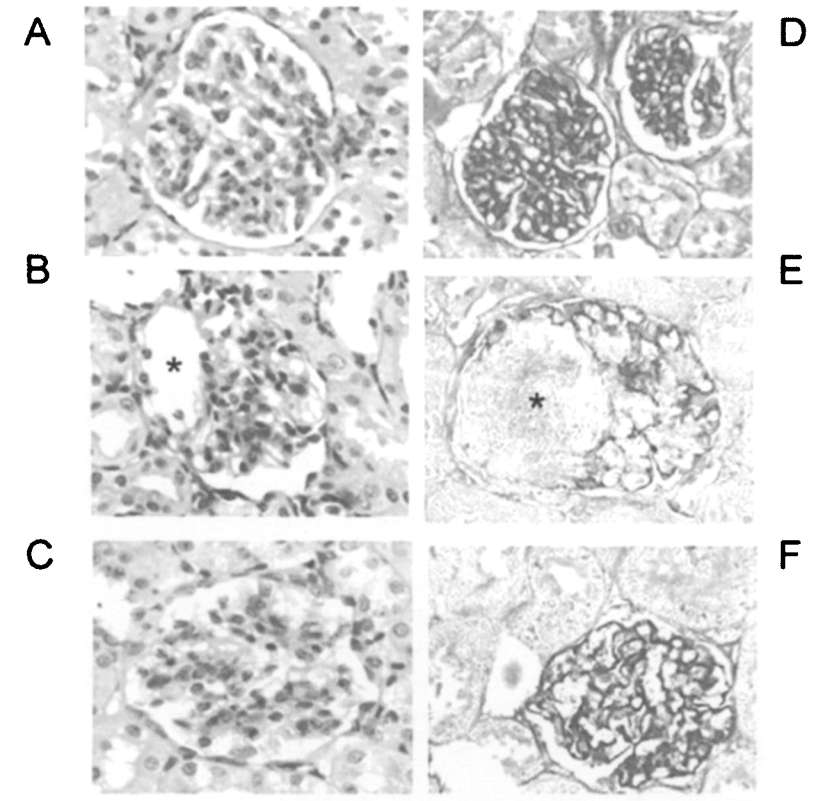


Fig. 2. *Glomerular morphology and glomerular polyanions distribution at day 7. PAS staining of a representative glomerulus of: (A) normal control rats (group III), (B) nephritic rats (group I) and, (C) group II rats treated with 1,25(OH)$_2$D$_3$. Colloidal iron staining of GPA of a representative glomerulus of: (D) normal control rats (group III), (E) nephritic rats (group I) and, (F) group II rats treated with 1,25(OH)$_2$D$_3$. Group I showed areas of mesangiolysis and mesangial proliferation with expansion of mesangial matrix and some aneurisms (*) of the glomerular capillary loop (B and E). In group II, 1,25(OH)$_2$D$_3$ administration protects from glomerular damages (C). The colloidal iron staining of GPA was reduced in kidneys of group I at day 7 especially in the correspondence of aneurisms of glomerular capillary loops (E). Original magnification: X 400 (A-F).*

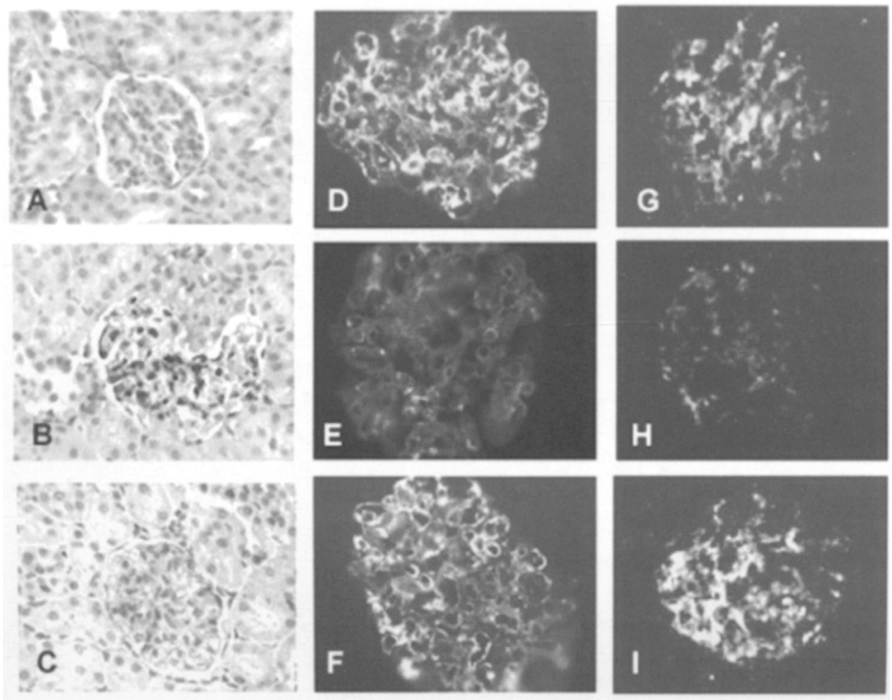


Fig. 3. *Micrographs representative of glomerular staining for desmin, nephrin and ZO- 1. Desmin expression detected by immunoperoxidase at day 7 in glomerular epithelial cells was enhanced in group I nephritic rats (B) in respect to group III controls (A). When nephritic rats where treated with 1,25(OH)$_2$D$_3$ (group II) glomerular staining for desmin was similar to that of controls (C). Immunofluorescence staining for nephrin (DF) and ZO-1 (H-I) was reduced in group I (E and H) in respect to group III (D and G). When nephritic rats where treated with 1,25(OH)$_2$D$_3$ (group II) glomerular staining for nephrin (F) and ZO-1 (I) was conserved. Original magnification: X 400 (A-I).*

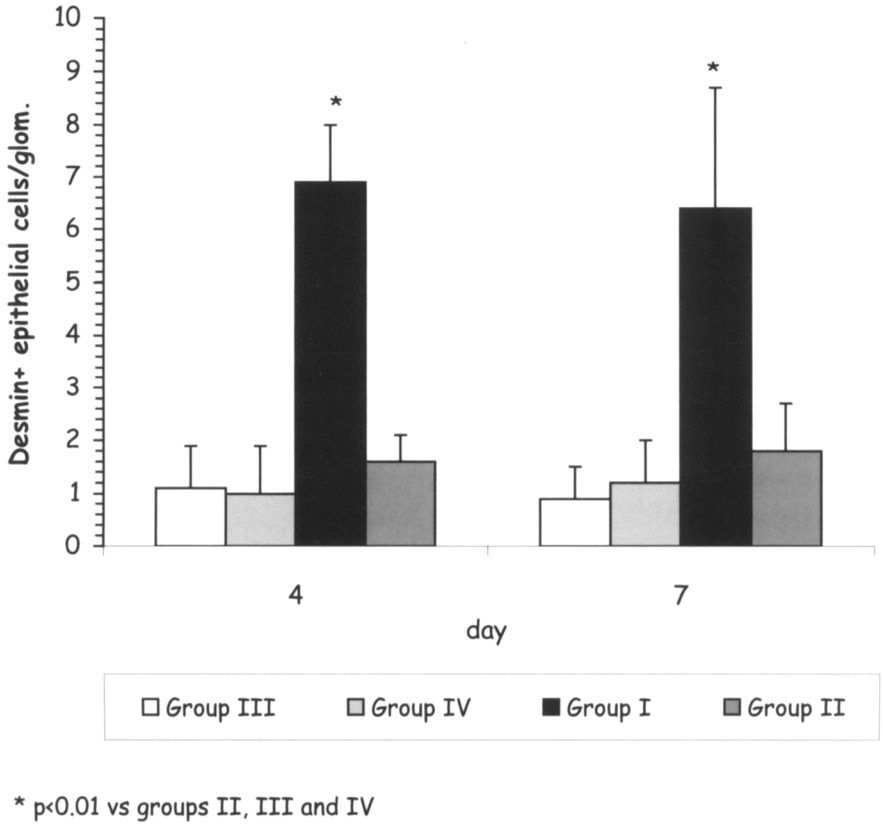


Fig. 4. *Number of desmin positive cells per glomerular cross section in different experimental groups at days 4 and 7. Results are expressed as mean ± SD; ANOVA with Dunnet's multicomparison test was performed among group I vs. groups II, III and IV (* P < 0.01).*

proteinuria was maintained until the end of the experimental period with a pick at day 7. In MPGN rats treated with 1,25-$(OH)_2D_3$ we also found a similar proteinuria at day 2 (before starting the drug administration). However, the day after the beginning of treatment with 1,25-$(OH)_2D_3$ (day 3), protein excretion was reduced compared to MPGN rats, reaching the values observed in normal controls from day 4 to the end of the study.

In MPGN rats morphological analysis revealed glomerular damage at day 4 and 7 with areas of mesangiolysis and mesangial proliferation with expansion of mesangial matrix (Fig. 2B) and some aneurism of the glomerular capillary loop (Fig. 2B and 2E). The colloidal iron staining of GPA was reduced especially in the correspondence of the aneurisms of the glomerular capillary loops (Fig. 2E) with respect to controls (Fig. 2D). Glomerular injury involved also the podocytes: as shown in figures 3 and 4, *de novo* expression of desmin in podocytes was significantly enhanced both at day 4 and 7. Slight diaphragm associated protein expression was also lost in damaged podocytes: fluorescence intensity for nephrin (Fig. 3 and 5A) was significantly reduced at day 4 and 7, whereas fluorescence intensity for ZO-1 (Figures 3 and 5B) was significantly reduced only at day 7.

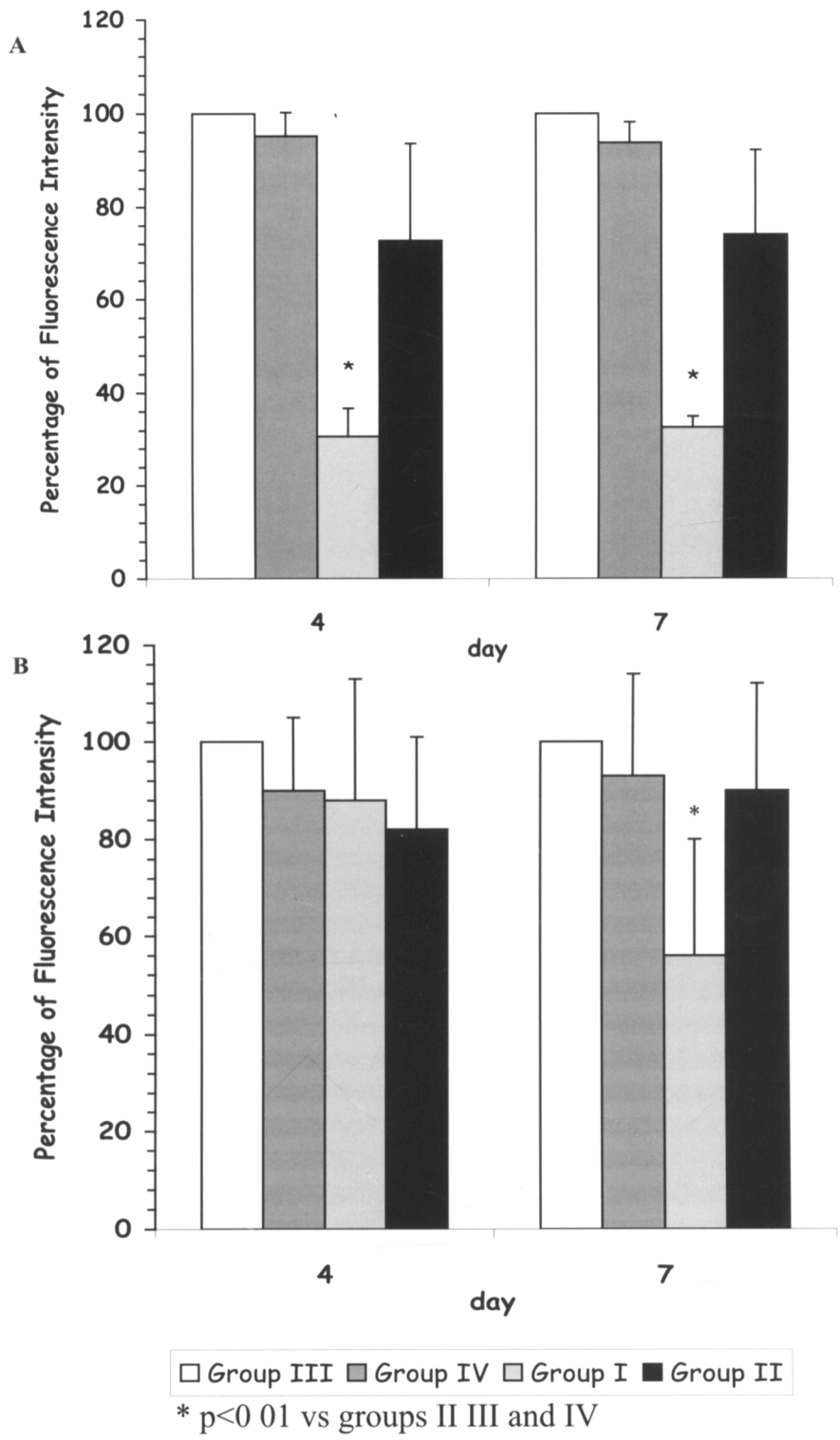


Fig. 5. *Semiquantitative analysis of nephrin (A) and ZO-1 (B) expression as detected by immunofluorescence staining in glomeruli of experimental groups at days 4 and 7. Results are expressed as percentage of control (group III, normal control rats). ANOVAwith Dunnet's multicomparison test was performed among group I vs. groups II, III, and IV (*P < 0.01).*

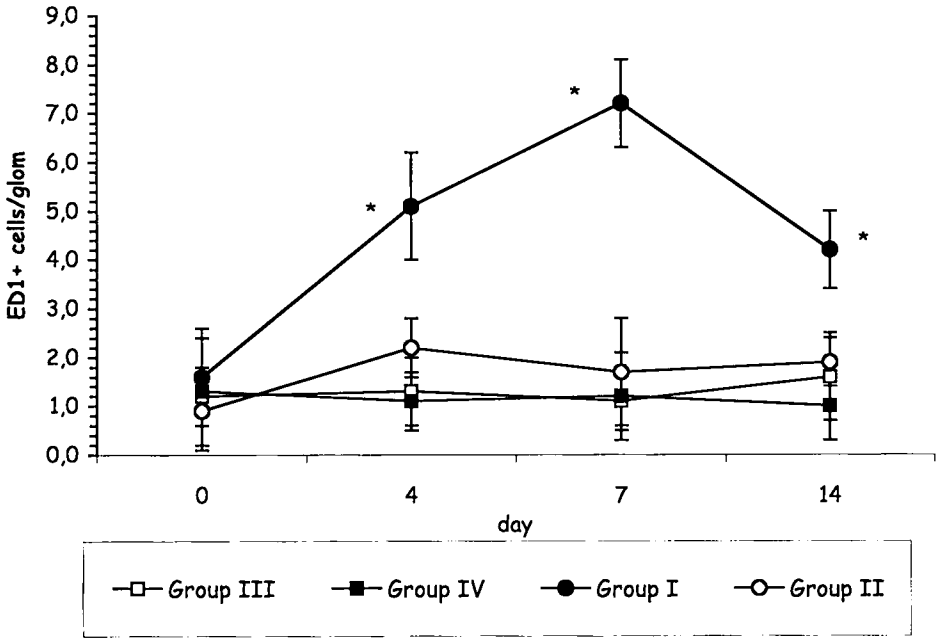


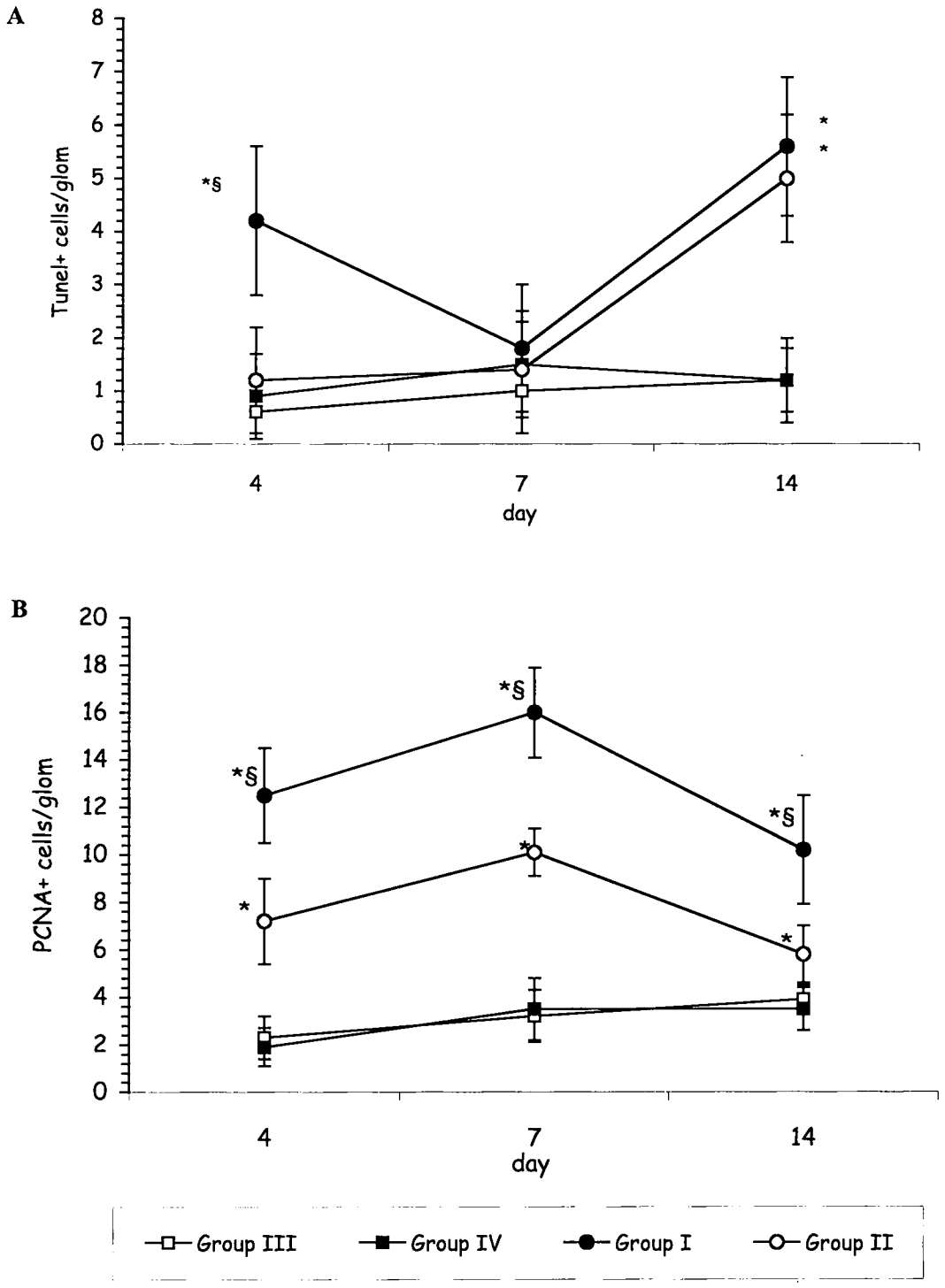
Fig. 6. *Number of ED-1 positive cells per glomerular cross section in different experimental groups at days 4, 7 and 14. Results are expressed as mean ± SD; ANOVA with Dunnet's multicomparison test was performed among group I vs. groups II, III, and IV (* P < 0.01).*

Consistent with the proteinuria time-course, $1,25(OH)_2D_3$ administration reduced glomerular damage (Figure 2C) and restore a normal staining for GPA (Figure 2F). Moreover, desmin staining was similar to normal controls both at day 4 and at day 7 (Fig. 3 and 4), and, as shown in figures 3 and 5A and B, treatment with $1,25\text{-}(OH)_2D_3$ significantly prevented the loss of nephrin and ZO-1 that was present in MPGN rats.

The number of ED1-positive monocytes was significantly increased at day 4, peaked at day 7 and decreased thereafter (Fig. 6). Treatment with $1,25(OH)_2D_3$ completely abrogated the glomerular accumulation of ED1-positive monocytes observed at days 4 and 7 (Figure 6). In MPGN rats, a first peak of apoptotic cells was observed at day 4 concomitantly with morphological aspects of mesangiolysis (Fig. 7A). Apoptosis was quite absent at day 7, but a second peak was detected at day 14 (Fig. 7A) concomitantly with the reduction of mesangial cell proliferation revealed by glomerular staining for PCNA (Fig. 7B). Intriguinly, the first peak of glomerular apoptosis was absent in MPGN rats treated with $1,25(OH)_2D_3$ (Fig. 7A). At variance, no differences in glomerular apoptosis were detected between these groups of animals at day 14. Mesangial proliferation was detected from day 4, peaked at day 7 and decreased thereafter. The extent of proliferation was reduced overall in drus-threated nephritic rats (Figure 7B).

DISCUSSION

The results of the present study demonstrate that in the model of anti-Thy-1.1 induced GN proteinuria was associated with podocyte injury and loss of slit diaphragm-associated proteins, and that these alterations were inhibited by treatment with $1,25(OH)_2D_3$ started after induction of the glomerular disease. Various conditions may cause glomerular injury and evoke a local wound-healing response. The mesangialproliferative glomerulonephritis induced by a single injection of mAb against Thy-1.1 in female Wistar rats represents a well-established



* p<0.01 vs Group III and Group IV; §p<0.05 vs Group II

Fig. 7. *Number of TUNEL positive cells (A) and PCNA positive cells (B) per glomerular cross section in different experimental groups at days 4, 7, and 14. ANOVA with Newman-Keul's multicomparison test was performed among group I vs. group III and IV (* $P < 0.01$), and group I vs. groups II (§ $P < 0.05$).*

experimental model to investigate the renal response to acute immune-mediated injury (24). The complement-dependent reactive oxygen species generation and the reduced scavenger activity of nitric oxide is one of the critical initiating event that follows fixation of anti-Thy 1 antibody (28-29). As previously reported (24), the administration of a single dose of anti-Thy-1.1 antibody in the rat induces a mesangial proliferative GN characterized by mesangiolysis, early accumulation of neutrophils and monocytes followed by mesangial proliferation and matrix expansion. The recovery was associated with apoptosis of inflammatory and mesangial cells in excess, capillary angiogenesis and matrix remodeling (30-31). Proteinuria underlines the course of glomerular injury increasing up to 7-10 days following anti-Thy-1.1 injection to decrease thereafter (24). We have demonstrated that pre-treatment with $1,25(OH)_2D_3$ was able to prevent the glomerular inflammatory injury and the onset of proteinuria (8).

In the present study, proteinuria was reverted by a "therapeutic" administration of $1,25(OH)_2D_3$ (i.e. started after its "clinical" evidence). Therefore, we investigated this anti-proteinuria effect by studying the potentially implicated mechanisms. It has been described that $1,25(OH)_2D_3$, such as linoleic acid (32) and all trans-retinoic acid (33), has an anti-inflammatory (1, 34-35) and anti-proliferative effects (36-38) and it could regulate the common mucosal immune responses to bacterial-derived substances that is consider disregulated in patients affected by mesangial-proliferative glomerulonephritis (39-41) . However, the observation that $1,25(OH)_2D_3$ inhibits proteinuria also in the model of Heymann GN (5), a non-inflammatory and proliferative GN, suggests that its effect is not merely anti-inflammatory. In the present study, we observed a positive staining for desmin, a marker of myogenic cells that is absent in mature podocytes (42) and a loss of colloidal iron staining of glomerular capillary walls, in MPGN rats. The staining of desmin, which is considered a marker of podocyte injury (42), was already evident at day 4 and persisted at day 7. The loss of GPA induced by anti-Thy-1.1 GN may reflect a reduction of glomerular polyanions of both cell glycocalix and glomerular basement membrane. Several studies have suggested that cationic inflammatory mediators may affect GPA expression (43, 44). The treatment with $1,25(OH)_2D_3$ significantly reduced glomerular staining for desmin and the loss of GPA. These effects may depend either on the anti-inflammatory activity of $1,25(OH)_2D_3$ or on a direct action on podocytes. Podocyte damage was concomitant with the loss of ZO-1 and nephrin, two slit diaphragm-associated-proteins. Recent studies have focused on the pivotal role of nephrin in the regulation of glomerular size-selective permeability, showing that mutations in nephrin gene underline the development of congenital Finnish type nephrotic syndrome (11, 45). A correlation between changes in nephrin expression and proteinuria has been found in several experimental models of GN (12-13, 46). Moreover, a redistribution and reduction of nephrin have been described in patients with primary acquired nephrotic syndrome and in diabetic nephropathy (18-22, 47). In our study, we detected a significant reduction in glomerular staining for nephrin in concomitance with the peak of proteinuria observed in anti-Thy-1.1 rats. Inflammatory mediators and hemodynamic factors generated in anti-Thy-1.1 GN, such as TNF-α, membrane attack complement and Ang II may have induced the loss of nephrin. Indeed, these mediators were show to induce *in vitro* redistribution and shedding of nephrin from the surface of podocytes (19, 22, 48). Treatment with $1,25(OH)_2D_3$ inhibited the loss of nephrin and ZO-1 in anti-Thy-1.1 GN concomitantly with inhibition of proteinuria and glomerular inflammatory reaction. These results suggest that $1,25(OH)_2D_3$ inhibits the alterations of podocyte occurring during the development of anti-Thy-1.1 GN. Consistently with this observation, $1,25(OH)_2D_3$ has been shown to decrease podocyte loss and podocyte hypertrophy in the subtotally nephrectomized rat (49).

In conclusion, the results of the present study suggest that $1,25(OH)_2D_3$ administrated with a therapeutic regiment may revert the early inflammatory injury and proteinuria, and protect podocyte from the dedifferentiation and loss of slit diaphragm-associated proteins.

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