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# Effect of $1,25(OH)_2$ vitamin $D_3$ on glomerulosclerosis in subtotally nephrectomized rats

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**Effect of $1,25(OH)_2$ vitamin $D_3$ on glomerulosclerosis in subtotally nephrectomized rats.** In the past, there has been considerable concern that treatment with active vitamin D might accelerate progression independent of hypercalcemia and hypercalcuria. Nevertheless, $1,25(OH)_2D_3$ has known antiproliferative properties and has also been shown to inhibit renal growth. Since glomerular growth is a permissive factor for the development of glomerulosclerosis, we reasoned that $1,25(OH)_2D_3$ might even attenuate progression. To test this working hypothesis we performed two experiments of 8 and 16 weeks duration, respectively, to compare subtotally nephrectomized (SNX) rats treated with ethanol and SNX treated with $1,25(OH)_2D_3$. Control animals were sham operated and pair-fed with SNX animals. $1,25(OH)_2D_3$ (3 ng/100 g body wt/day) was administered by osmotic minipump. $1,25(OH)_2D_3$ had no significant effect on systolic blood pressure and only a transient effect on weight gain. SNX reduced the number of glomeruli (left kidney) from an average of $3.3 \times 10^4$ to $1.2 \times 10^4$ per kidney. Mean glomerular volume was $3.87 \pm 0.71 \times 10^6$ $\mu m^3$ in sham operated animals and significantly ($P < 0.05$) higher ($10.1 \pm 1.75 \times 10^6$ $\mu m^3$) in untreated animals 16 weeks after SNX. Glomerular volume was significantly ($P < 0.05$) less in $1,25(OH)_2D_3$ treated SNX [$10.1 \pm 1.75$ in ethanol vs. $7.04 \pm 1.78$ in $1,25(OH)_2D_3$ treated SNX]. In parallel, there was significantly ($P < 0.01$) less glomerulosclerosis [glomerulosclerosis index $1.16 \pm 0.14$ in the ethanol treated SNX vs. $0.80 \pm 0.16$ in SNX treated with $1,25(OH)_2D_3$] in the eight week experiment. Albuminuria was significantly ($P < 0.01$) lower in $1,25(OH)_2D_3$ treated than in ethanol treated SNX (mean 0.785 mg/24 hr, range 0.43 to 1.80, vs. 3.75 mg/24 hr, 1.29 to 14.2). The morphological data were directionally analogous in a second 16 week experiment. Only slight changes of the vascular sclerosis index and tubulointerstitial index were seen in SNX and were not affected by $1,25(OH)_2D_3$ further. To prove that the effect of $1,25(OH)_2D_3$ was independent of PTH, parathyreoidectomized SNX rats without or with $1,25(OH)_2D_3$ treatment were examined seven days post-SNX. PCNA staining showed suppression of cell proliferation. Furthermore, *in situ* hybridization for transforming growth factor-B (TGF-$\beta$) showed less vascular and tubular expression in $1,25(OH)_2D_3$ treated rats. We conclude that $1,25(OH)_2D_3$ has antiproliferative actions during the compensatory growth of nephrons in response to subtotal nephrectomy. These effects are independent of PTH. The data document that $1,25(OH)_2D_3$ reduces renal cell proliferation and glomerular growth as well as glomerulosclerosis and albuminuria as indicators of progressive glomerular damage.

**Key words:** renal growth, vitamin D, progressive renal disease, secondary hyperparathyroidism, albuminuria, renoprotective factor.

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After treatment with high doses of vitamin $D_3$ was introduced for uremic patients with disturbed calcium metabolism [1], more rapid progression of renal failure was noted in many patients. This was attributed to vitamin D toxicity as a result of hypercalcemia. While Christiansen, Rodbro and Christiansen [2] proposed that active vitamin D metabolites were nephrotoxic independently of hypercalcuria and hypercalcemia, this hypothesis has not been confirmed in several prospective controlled trials [3–7].

In view of such historical concerns about the potential intrinsic nephrotoxicity of active vitamin D, it is paradoxical that some recent experimental findings would be consistent even with a nephroprotective effect of $1,25(OH)_2D_3$. In numerous cell systems, such as, keratinocytes [8], chondrocytes [9], colonic carcinoma cells [10], lymphoma cells [11] or monocytoid cells U 937 [12] $1,25(OH)_2D_3$ was shown to inhibit cell proliferation and to promote cell differentiation. Apparently this also applies to the kidney. In a renal cell carcinoma line, Nagakura et al [13] showed a dose-dependent inhibition of proliferation by $1,25(OH)_2D_3$. Examining renal compensatory growth after uninephrectomy, *in vivo* experiments of Matthias et al [14] documented that $1,25(OH)_2D_3$ reduced renal weight, renal protein content, renal DNA content and the number of mitoses. These effects were independent of PTH secretion. Finally, at least *in vitro*, both in human mesangial cells [15] and in a proximal tubular cell line [16] $1,25(OH)_2D_3$ diminished radiothymidin incorporation, cell counts and TGF-$\beta$ secretion into the supernatant [17]. Such antiproliferative actions of $1,25(OH)_2D_3$ may be related to the recently described activation of the Cdk inhibitor p21 by the active vitamin D metabolite [18].

It is commonly assumed that glomerular growth is a factor that favors progression of glomerulosclerosis [19]. This hypothesis has provided the rationale to examine whether $1,25(OH)_2D_3$ exerted a beneficial effect in the renal ablation model. To this end we compared solvent treated and $1,25(OH)_2D_3$ treated subtotally nephrectomized rats. We measured glomerular morphology by stereological techniques, assessed cell proliferation using the PCNA technique, analyzed expression of the sclerosis promoting cytokine TGF-$\beta$ using non-radioactive *in situ* hybridization, and assessed urinary albumin excretion using immune detection. To exclude the possibility that the effect of $1,25(OH)_2D_3$ was mediated via suppression of PTH, treatment with $1,25(OH)_2D_3$ was also assessed in parathyroidectomized SNX rats.

## METHODS

### Animals

Male 160 to 180 g Sprague Dawley rats (SD) were housed in single cages at constant room temperature (20°C) and humidity (75%) under a controlled light/dark cycle. The rats were fed a diet containing a 20% protein, 0.9% calcium/100 g and 600 IE vitamin $D_3$ per kg. The animals had free access to drinking water.

### Surgery

After a three-day adaptation period, the animals were randomly allotted to sham operation or subtotal nephrectomy (SNX). For subtotal nephrectomy the right kidney was removed in a first session. Eight days after the first operation 75% of cortical tissue by weight was removed surgically under anesthesia with Ketanest (100 mg/kg body wt) and Rompun (2 mg/kg body wt). In sham operated animals, both kidneys were decapsulated in two sessions. For parathyroidectomy (PTX) the parathyroids were removed using microsurgical techniques.

### Protocol

Sham operated and subtotally nephrectomized (SNX) rats were pair-fed, that is, control animals received the amount of food consumed by the matched SNX animals on the preceeding day. The animals were given $1,25(OH)_2D_3$ or ethanol by osmotic minipump. The dose of $1,25(OH)_2D_3$ was 3 ng/100 g body wt/day. A pilot study showed that in non-SNX rats treated with this dose, serum calcium ($S_{Ca}$) did not exceed 2.51 mmol/liter. Administration was via a Savo Company minipump model 2 ml 4 (Kisslegg, Allgäu, Germany) at an hourly delivery rate of 2.5 $\mu$l.

Two main experiments were carried out. In the first experiment, animals were studied for eight weeks comparing (*i*) sham-operated solvent treated, (*ii*) sham-operated $1,25(OH)_2D_3$ treated, (*iii*) SNX ethanol treated, and (*iv*) SNX $1,25(OH)_2D_3$ treated rats. In each group, there were at least 8 to 12 animals. In the second experiment with the same protocol, animals were studied for 16 weeks.

In the above two experiments, ethanol treated sham-operated animals were pair-fed with ethanol treated SNX; the $1,25(OH)_2D_3$ treated sham-operated animals were pair-fed with $1,25(OH)_2D_3$ treated SNX. In these two series the kidneys were examined by morphological techniques. Since food intake had been somewhat lower in $1,25(OH)_2D_3$ treated animals, in a repeat of the eight week experiment the SNX ethanol treated animals were pair-fed with SNX $1,25(OH)_2D_3$ treated animals to exclude a lower food intake in $1,25(OH)_2D_3$ treated animals as a confounding factor. All other conditions were kept identical. In this latter experiment parameters of calcium metabolism and urinary albumin excretion were measured.

In ancillary studies animals were treated for immunhistochemical and molecular biological examination. The experimental details are described below.

### Measurements

Body weight and serum calcium were measured at regular intervals.

At the end of the experiment, blood samples were taken for routine chemistry (Autoanalyzer, Fa. Hitachi, Japan). Systolic blood pressure was measured by tail cuff plethysmography. The experiment was terminated by retrograde perfusion fixation via the abdominal aorta as described in detail elsewhere [20]. $1,25(OH)_2D_3$ was measured immunoradiometrically as described elsewere [21]. Urinary albumin was measured using the microplate technique described by Magnotti et al [22], modified by using a rabbit anti-rat albumin peroxydase conjugate.

### Tissue preparation

The kidneys were taken out, weighed and then dissected in a plane perpendicular to the interpolar axis, yielding slices of 1 mm width. Ten small pieces of each kidney were selected by area weighted sampling [20] for embedding in Epon-Araldite. The remaining tissue slices were embedded in paraffin; 4 $\mu$m sections were taken and stained with hematoxylin/eosin (HE) and PAS. Subsequently, the kidneys were investigated by means of morphometry and stereology [23, 24].

For immunohistological investigations (PCNA) one half of the kidney was fixed in 8% buffered formaldehyde. For *in situ* hybridization, the other half of the kidney was snap-frozen in liquid nitrogen-cooled isopentane. Cryostat sections 12 $\mu$m in thickness were thaw-mounted on silane-coated slides and fixed for five minutes in 3% paraformaldehyde/phosphate buffered saline (PBS), pH 7.0. Deproteination was performed in 0.2 M HCl. To reduce the nonspecific background, the sections were acetylated for 20 minutes in 0.25% acetic acid (vol/vol) in 0.1 M triethanolamine, pH 8.0. After dehydration in graded alcohols, the sections were allowed to air-dry prior to prehybridization.

### Immunohistological staining

For PCNA staining an anti-PCNA antibody (Immunotech 1510 mono, Marseille, France) was used in a 1:150 dilution. The concentration that was optimal for staining was evaluated testing different dilution-series in a pilot study.

To avoid nonspecific reactions (cross reaction) of tissue components with antibody, a biotin-streptavidin detection system (biotin-streptavidin super sensitive; Fa. BioGenex, San Ramon, CA, USA) was selected for staining the specimen. With this system nonspecific cross reactions can be excluded. Furthermore, a high sensitivity can be achieved even with short incubation times. The following immunhistochemical protocol was used: 15 minutes TUF (90°C), 60 minutes primary antibody, 3 × 5 minutes TBS pH 7.6, 30 minutes anti-PCNA antibody (Immunotech 1510 mono diluted 1:150), 3 × 5 minutes TBS, pH 7.6, 30 minutes Label (super sensitive; Fa. BioGenex), 30 minutes Levamisole 0.2 g, and 100 ml TBS, pH 7.6, fast red substrate system (DAKO), Mayers hämalaun.

### *In situ* hybridization

*Probe preparation and labeling*. A 404 bp cDNA encoding positions 1.156 to 1.559 of the rat TGF-$\beta$1 gene [25] was inserted into the expression vector pGEM-5Zf(+) (Promega Biotech., Madison, WI, USA). To generate an antisense RNA probe, the plasmid was linearized with *Sal*I and transcribed using T7 RNA polymerase. For preparation of the sense RNA probe, the plasmid was linearized with *Nco*I and transcribed using SP6 RNA polymerase.

Digoxigenin-labeled RNA probes were synthesized with a DIG RNA labeling mix (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, labeled transcripts were generated in 20 $\mu$l transcription buffer [40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 10 mM dithiothreitol

(DTT), 2 mM spermidine] containing 1 μg agarose gel-extracted linearized plasmid, NTP-labeling mix (1 mM each of ATP, CTP and GTP, 0.65 mM UTP, 0.35 mM DIG-UTP), 20 U of RNAsin ribonuclease inhibitor and 40 U of T7 and SP6 RNA polymerase, respectively. The reaction was stopped by digestion of the DNA template with RNase-free DNase and the labeled transcripts were ethanol-precipitated at −20°C overnight. Subsequently, the probes were pelleted and resuspended in DEPC-treated water. The probe concentration was determined in a UV-spectrophotometer at 260 nm. Incorporation efficiency of digoxigenin-11-d-UTP into the probe and probe length were estimated by running an aliquot on a denaturating formaldehyde gel, blotting it onto a nylon membrane and comparing the size and intensity of the colorimetric reaction from a diluted, labeled, control RNA (Boehringer Mannheim).

*In situ hybridization*. The fixed sections were prehybridized for four hours at 38°C with 50% deionized formamide, 50 mM Tris-HCl, pH 7.6, 25 mM EDTA, pH 8.0, 0.625 mg/ml yeast tRNA, 2.5 × Denhardt's solution [0.05% Ficoll 400, 0.05% polyvinylpyrollidone, 0.05% bovine serum albumine (BSA)], and 20 mM NaCl in a humidified chamber. Then, 120 ng labeled probe per section in hybridization buffer (50% deionized formamide, 20 mM Tris-HCl, pH 7.6, 0.33 M NaCl, 0.1 M DTT, 10% dextran sulfate, 0.5 mg/ml yeast tRNA, 0.1 mg/ml poly-A-RNA, 1× Denhardt's solution) was applied to the sections. Hybridization of the probe with the native mRNA was allowed for 18 hours at 38°C.

Post-hybridization steps included removal of coverslips in 1 × standard saline citrate (SSC) at 48°C, two subsequent washes in 0.5 × SSC/50% formamide for one hour at 48°C, RNase A treatment (10 μg/ml) in 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTAm pH 7.5 for 30 minutes at 37°C, and washes for 10 minutes at 48°C in 1 × SSC, 0.5 × SSC and 0.25 × SSC, respectively.

*Immunohistological detection of hybridized probes*. After post-hybridization washes, the sections were equilibrated in dig-buffer I (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5) and blocked by incubation for one hour with 1% normal sheep serum. Alkaline phosphatase-conjugated sheep anti-digoxigenin-Fab fragments (750 U/ml), diluted 1:500 in dig-buffer containing 0.3% Triton X100 and 1% normal sheep serum were applied to each slide and incubated overnight at 4°C. Unbound conjugate was removed by two 10 minutes washes in dig-buffer I followed by equilibration of the sections in dig-buffer II (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). The detection of the signal was performed by incubation at 4°C in darkness for up to 40 hours in a chromogen solution containing 0.375 mg/ml nitro blue tetrazoliumchloride (NBT) and 0.188 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in dig-buffer II. At optimal color development, the reaction was halted by immersing the slides in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Subsequently, the slides were counterstained with neutral red and visualized by light microscope.

### Morphological investigations

*Indices of renal damage (glomerular sclerosis, tubulointerstitial and vascular damage)*. The total area of sclerosis within the glomerular tuft as an index of progression was determined adopting the semiquantitative scoring system proposed by El Nahas et al [26]. Using light microscopy, a glomerular score was derived for each animal by examining 100 glomerula at a magnification of ×400. The severity of scarring was expressed on an arbitrary scale from 0 to 4. The glomerular score for individual glomerula was: grade 0, normal glomerula; grade 1, presence of mesangial expansion/thickening of the basement membrane; grade 2, mild/moderate segmental hyalinosis/sclerosis involving less than 50% of the glomerular tuft; grade 3, diffuse glomerular hyalinosis/sclerosis involving more than 50% of the tuft; grade 4, diffuse glomerulosclerosis with total tuft obliteration and collapse. The resulting index in each animal was expressed as a mean of all scores obtained.

A tubulointerstitial score (0 to 4) and vascular changes (0 to 3) was also used: tubulointerstitial (histological expressed in tubular atrophy, dilation, casts, interstitial inflammation, and fibrosis) and vascular changes were assessed using the semiquantitative scoring system proposed by Véniant et al [27]. In PAS stained paraffin sections at a magnification of ×100, ten fields per kidney were randomly sampled and graded as follows: grade 0, no changes; grade 1, lesions involving less than 25% of the area; grade 2, lesions affecting 25 to 50%; grade 3, lesions involving more than 50% and grade 4 involving (almost) the entire area.

*Glomerular geometry*. Area ($A_A$) and volume density ($V_V$) of the renal cortex and medulla as well as the number of glomeruli per area ($N_A$) were measured using a Zeiss eyepiece (Integrationsplatte II; Zeiss Co., Oberkochen, Germany) and the point counting method ($P_P = A_A = V_V$) at a magnification of ×400 [23, 24]. The number of glomeruli per area ($N_A$) was then corrected for tissue shrinkage (45%).

Total cortex volume ($V_{cortex}$) was derived from kidney mass devided by specific weight of the kidney (1.04 g/cm$^3$) times the volume density of the cortex. Glomerular geometry was analyzed as follows: Volume density ($V_V$) of glomerula and area density of glomerular tuft ($A_{AT}$) were measured by point counting according to $P_P = A_A = V_V$ [23, 24] at a magnification of ×400 on HE sections.

Total area of glomerular tuft ($A_T$) was then determined as

$$A_T = A_{AT} \times A_{cortex}$$

The number of glomerula per volume ($N_V$) was then derived from glomerular area density ($N_A$) and the volume density ($V_V$) of glomerula using the formula:

$$N_V = k/\beta \times N_A^{1.5}/V_V^{0.5}$$

with $k = 1$ and $\beta = 1.382$.

The total number of glomeruli was derived from the total volume of the renal cortex and the number of glomeruli per cortex volume:

$$N_{glom} = N_V \times V_{cortex}$$

The mean glomerular tuft volume was determined according to

$$v = \beta/k \times A_T^{1.5}$$

with $\beta = 1.382$ and $k = 1$ [23, 24].

*Immunohistological investigations*. The tissue sections stained for PCNA positive cells were examined using light microscopy at a magnification of ×400. Fifty glomeruli per kidney were selected and the number of PCNA-positive cells were counted.

### Statistics

Data are given as mean ± SD. The Kruskal-Wallis test and one-way ANOVA respectively were chosen for analysis of variance, followed by Ducan's multiple-range test to determine

**Table 1.** Animal data

| | Experiment 1 (8 weeks) | | | | | | Experiment 2 (16 weeks) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *N* | Body wt *g* | Systolic BP[a] *mm Hg* | LV/body wt ratio [$\times 10^{-3}$] | $S_{Cr}$ *mg/dl* | $S_{Ca}$ *mg/dl* | *N* | Body wt *g* | Systolic BP[a] *mm Hg* | LV/body wt ratio [$\times 10^{-3}$] | $S_{Cr}$ *mg/dl* | $S_{Ca}$ *mg/dl* |
| Sham operated + ethanol | 12 | 488 ± 34.0[c] | 116 ± 11.3 | 1.88 ± 0.11 | 0.65 ± 0.88 | 2.45 ± 0.34 | 10 | 467 ± 23.0 | 114 ± 8.67 | 2.34 ± 0.29 | 0.91 ± 0.10 | 2.28 ± 0.14 |
| Sham operated + $1,25(OH)_2D_3$ | 12 | 436 ± 38.9 | 123 ± 12.1 | 1.90 ± 0.14 | 0.66 ± 0.10 | 2.50 ± 0.27 | 10 | 471 ± 27.2 | 119 ± 9.70 | 2.30 ± 0.40 | 0.92 ± 0.09 | 2.39 ± 0.09 |
| Subtotal nephrectomy + ethanol | 11 | 456 ± 58.8[d] | 122 ± 12.7 | 2.49 ± 0.37[bc] | 0.95 ± 0.07[bc] | 2.58 ± 0.14[bc] | 12 | 487 ± 33.7[b] | 131 ± 11.1[bc] | 2.44 ± 0.38 | 1.50 ± 0.25[bc] | 2.36 ± 0.13 |
| Subtotal nephrectomy + $1,25(OH)_2D_3$ | 8 | 412 ± 38.0[b] | 129 ± 25.7 | 2.43 ± 0.31[bc] | 0.89 ± 0.10[bc] | 2.67 ± 0.20[bc] | 13 | 465 ± 22.4 | 130 ± 18.6[b] | 2.38 ± 0.30 | 1.63 ± 0.14[bc] | 2.43 ± 0.07 |
| *P*, Analysis of variance | | < 0.05 | NS | < 0.05 | < 0.05 | < 0.05 | | < 0.05 | < 0.05 | NS | < 0.05 | NS |

Abbreviations are: LV, weight of perfusion-fixed left ventricle; $S_{Cr}$, serum creatinine; $S_{Ca}$, serum calcium.
[a] At the end of the experiment
[b] $P < 0.05$ vs. sham operated rats treated with ethanol
[c] $P < 0.05$ vs. sham operated rats treated with $1,25(OH)_2D_3$
[d] $P < 0.05$ vs. SNX rats treated with $1,25(OH)_2D_3$

whether or not the differences between the groups were significant. The results were considered significant when the probability of error (*P*) was less than 0.05.

## RESULTS

### Animal data

Table 1 lists the pertinent data in experiment 1 (8 weeks duration) and experiment 2 (16 weeks).

It is of note that in the short-term study body weight was significantly lower in $1,25(OH)_2D_3$ treated animals; this was not true for the long-term study. Because of the pair-feeding protocol, food consumption was identical in the SNX + ethanol and sham operated + ethanol-treated animals, that is, 234 ± 32.4 g per eight days, and in the SNX + $1,25(OH)_2D_3$ and sham operated + $1,25(OH)_2D_3$ treated animals, that is, 214 ± 31.3 g per eight days.

Treatment with $1,25(OH)_2D_3$ had no consistent effect on systolic blood pressure (BP) at the end of the experiment, on left ventricle (LV) weight or the LV/body wt ratio. Treatment with $1,25(OH)_2D_3$ caused a modest increase of serum calcium within the normal range.

In ethanol treated SNX rats the relative weight of the left kidney remnant (4.54 ± 0.55 g/g body wt $\times 10^{-3}$) was significantly higher than the left kidney weight/body wt ratio of sham operated controls (3.65 ± 0.28 g/g body wt $\times 10^{-3}$). Treatment with $1,25(OH)_2D_3$ had no significant effect [4.98 ± 0.45 in SNX + $1,25(OH)_2D_3$ and 3.96 ± 0.41 in sham operated + $1,25(OH)_2D_3$; all data are from experiment 1].

### Short-term study (experiment 1)

As shown in Table 2, SNX reduced the number of glomeruli by approximately 70%. In untreated animals the mean glomerular volume increased on average by a factor of 2. Total glomerular volume (that is, conceptually the volume of all glomeruli per kidney or kidney remnant combined) was only slightly decreased as a result of a compensatory increase in the mean volume of individual glomerula.

In ethanol treated SNX the glomerulosclerosis index was significantly increased. In rats treated with $1,25(OH)_2D_3$ the glomerulosclerosis index was lower, both in sham operated and SNX (Table 2 and Fig. 1).

Treatment with $1,25(OH)_2D_3$ had no significant influence on the vascular sclerosis index (SNX + ethanol 1.08 ± 0.13; SNX + $1,25(OH)_2D_3$ 1.19 ± 0.14) or tubulointerstitial index (SNX + ethanol 1.30 ± 0.22; SNX + $1,25(OH)_2D_3$ 1.28 ± 0.30).

The relative contribution of cortex and medulla in coronal sections of the kidney remnant was normalized after eight weeks (and 16 weeks; Table 3). A marginal effect was seen with $1,25(OH)_2D_3$ treatment.

In a repeat eight week experiment comprised of subtotally nephrectomized rats, a pair-feeding protocol was used (Table 4). Ethanol and $1,25(OH)_2D_3$ treated animals were compared. Body weight in $1,25(OH)_2D_3$ treated animals was again significantly lower than in ethanol controls despite the identical food intake. Serum calcium concentration and urinary calcium excretion were only modestly increased. Plasma $1,25(OH)_2D_3$ concentrations documented adequate delivery of the secosterole by the osmotic minipump. Albumin excretion rates were significantly higher in ethanol compared to $1,25(OH)_2D_3$ treated animals. With the exception of one pair, there was no overlap of values.

### Long-term study (experiment 2)

In the long-term experiment with a similar reduction of the number of glomeruli, a more marked increase in mean glomerular volume was noted (Table 2). The glomerulosclerosis index was also higher in the ethanol treated animals compared to experiment 1. Again, a significant reduction of the mean glomerular volume and glomerulosclerosis index was seen in SNX treated with $1,25(OH)_2D_3$ (Table 2 and Fig. 1). As in experiment 1, no

**Table 2.** Glomerular measurements

| | Experiment 1 (8 weeks) | | | | | Experiment 2 (16 weeks) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *N* | Glomerulo-sclerosis index | Total glomerular volume [cm$^3$] per kidney | Total number of glomeruli | Mean volume of individual glomerula ($\times 10^6 \mu m^3$) | *N* | Glomerulo-sclerosis index | Total glomerular volume [cm$^3$] per kidney | Total number of glomeruli | Mean volume of individual glomeruli ($\times 10^6\ \mu m^3$) |
| Sham operated + ethanol | 12 | 0.41 ± 0.08[b] | 0.104 ± 0.01 | 27511 ± 3646 | 3.81 ± 0.54 | 10 | 0.54 ± 0.07 | 0.11 ± 0.01 | 29094 ± 3855 | 3.87 ± 0.71 |
| Sham operated + 1,25$(OH)_2D_3$ | 12 | 0.23 ± 0.08 | 0.102 ± 0.02 | 33258 ± 4584[a] | 2.70 ± 0.45 | 10 | 0.51 ± 0.06 | 0.10 ± 0.02 | 30976 ± 5685 | 3.28 ± 0.64 |
| Subtotal nephrectomy + ethanol | 11 | 1.16 ± 0.14[abc] | 0.089 ± 0.01[ab] | 11985 ± 1763[ab] | 8.50 ± 1.61[ab] | 12 | 1.25 ± 0.17[abc] | 0.14 ± 0.03[abc] | 13840 ± 3401[ab] | 10.1 ± 1.75[ab] |
| Subtotal nephrectomy + 1,25$(OH)_2D_3$ | 8 | 0.80 ± 0.16[ab] | 0.089 ± 0.02[ab] | 11608 ± 1749[ab] | 7.99 ± 2.03[ab] | 13 | 0.93 ± 0.11[ab] | 0.10 ± 0.02 | 14179 ± 2178[ab] | 7.04 ± 1.78[abc] |
| *P*, Analysis of variance | | < 0.01 | < 0.05 | < 0.05 | < 0.05 | | < 0.001 | < 0.05 | < 0.05 | < 0.05 |

[a] $P < 0.05$ vs. sham operated rats treated with ethanol
[b] $P < 0.05$ vs. sham operated rats treated with 1,25$(OH)_2D_3$
[c] $P < 0.05$ vs. SNX rats treated with 1,25$(OH)_2D_3$

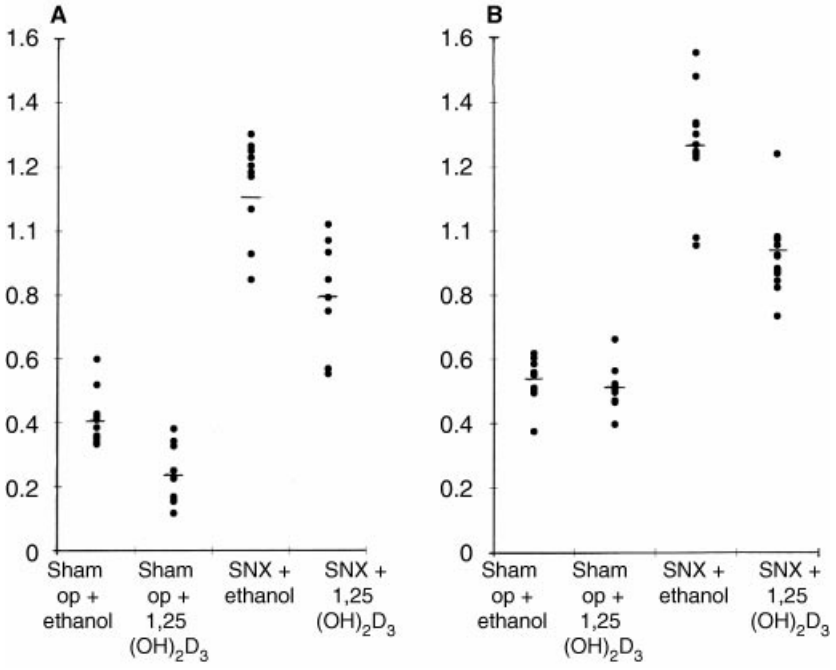


**Fig. 1. Median and range of individual glomerulosclerosis indices (*A*, 8 week experiment; *B*, 16 week experiment).**

significant effect of 1,25$(OH)_2D_3$ treatment was noted either on vascular damage index or the interstitial damage index (data not shown).

### Ancillary studies

The number of PCNA positive cells in PTX rats examined seven days after SNX was significantly lower with the 1,25$(OH)_2D_3$ treatment (Table 5). This was true for sham operated and for SNX animals. A representative example of the effect of 1,25$(OH)_2D_3$ treatment in SNX + PTX rats is shown in Figure 2.

An *in situ* hybridization study to assess the expression of TGF-$\beta$ in parathyroid intact SNX rats without or with 1,25$(OH)_2D_3$ treatment, studied seven days after SNX, showed considerably less expression of TGF-$\beta$ mRNA in animals treated with 1,25$(OH)_2D_3$. This was particularly noticeable in the vascular wall and in tubuloepithelial cells usually exhibiting the characteristics of proximal tubular cells. A representative example is shown in Figure 3. The expression in glomerular tissue was relatively low seven days after SNX.

## DISCUSSION

The above experiments were designed to test the working hypothesis that 1,25$(OH)_2D_3$ interfered with (*i*) glomerular proliferation and growth and (*ii*) development of glomerulosclerosis.

**Table 3.** Zonal analysis of kidney

| | Experiment 1 (8 weeks) | | | Experiment 2 (16 weeks) | | |
|---|---|---|---|---|---|---|
| | *N* | Cortex % | Medulla % | *N* | Cortex % | Medulla % |
| Sham operated + ethanol | 12 | 68.8 ± 1.32 | 31.2 ± 1.32 | 10 | 70.0 ± 2.99 | 30.1 ± 2.89 |
| Sham operated + $1,25(OH)_2D_3$ | 12 | 68.5 ± 3.46 | 31.5 ± 3.46 | 10 | 70.8 ± 3.58 | 29.2 ± 3.62 |
| Subtotal nephrectomy + ethanol | 11 | 64.8 ± 2.94[abc] | 35.8 ± 2.94[ab] | 12 | 68.1 ± 3.76 | 31.9 ± 3.78 |
| Subtotal nephrectomy + $1,25(OH)_2D_3$ | 8 | 69.4 ± 3.48 | 30.6 ± 3.48 | 13 | 70.1 ± 4.35 | 30.0 ± 4.40 |
| *P*, Analysis of variance | | < 0.05 | < 0.05 | | NS | NS |

[a] $P < 0.05$ vs. sham operated rats treated with ethanol
[b] $P < 0.05$ vs. sham operated rats treated with $1,25(OH)_2D_3$
[c] $P < 0.05$ vs. SNX rats treated with $1,25(OH)_2D_3$

**Table 4.** Calcium metabolism and albuminuria (8 week experiment)

| | *N* | Body weight *g* | $S_{Cr}$ *mg/dl* | $S_{Ca}$ *mg/dl* | $U_{Ca}V$ *μmol/24 hr* | Serum-$1,25(OH)_2D_3$ concentration *ng/ml* | $U_{Al}V$ *mg/24 hr* |
|---|---|---|---|---|---|---|---|
| Subtotal nephrectomy + ethanol | 10 | 396 ± 17.2 | 1.02 ± 0.15 | 2.68 ± 0.07 | 149 ± 50.9 | 22.3 ± 9.04[a] | 3.75[b] (1.29–14.2) |
| Subtotal nephrectomy + $1,25(OH)_2D_3$ | 9 | 365 ± 26.1 | 0.76 ± 0.08 | 2.71 ± 0.08 | 216 ± 59.7 | 69.0 ± 18.4 | 0.785 (0.43–1.80) |
| Mann-Whitney U test for pair differences, *P* | | < 0.05 | < 0.01 | NS | < 0.05 | < 0.01 | < 0.01 |

All data were collected at the end of the experiment (pair-feeding between animals with $1,25(OH)_2D_3$ and ethanol, respectively). Abbreviations are: $S_{Cr}$, serum creatinine; $S_{Ca}$, serum calcium; $U_{Ca}V$, urinary calcium excretion; $U_{Alb}V$, urinary albumin excretion.
[a] At the beginning of the experiment, 43.1 ± 10.3 ng/ml
[b] Given as median and range

**Table 5.** Number of PCNA positive cells in parathyroidectomized (PTX) rats examined 7 days after subtotal nephrectomy or sham operation, respectively

| | Number of PCNA positive cells | |
|---|---|---|
| | per glomerulum | per $mm^2$ of tubulointerstitial space |
| Sham operated + PTX + ethanol ($N = 10$) | 3.74 ± 1.99 | 17.5 ± 8.49 |
| Sham operated + PTX + $1,25(OH)_2D_3$ ($N = 10$) | 1.30 ± 1.31[a] | 8.18 ± 5.17 |
| SNX + PTX + ethanol ($N = 9$) | 4.90 ± 2.15[b] | 66.1 ± 23.0[abc] |
| SNX + PTX + $1,25(OH)_2D_3$ ($N = 9$) | 3.38 ± 1.61[b] | 26.5 ± 12.3[b] |
| *P,* Analysis of variance | < 0.05 | < 0.05 |

[a] $P < 0.05$ versus parathyroidectomized sham operated rats treated with ethanol
[b] $P < 0.05$ versus parathyroidectomized sham operated rats treated with $1,25(OH)_2D_3$
[c] $P < 0.05$ versus parathyroidectomized subtotally nephrectomized rats treated with $1,25(OH)_2D_3$

In order to avoid biostatistical artifacts from repetitive testing (Bonferroni problem) mean glomerular volume and glomerulosclerois index were predefined as the primary study endpoints. Statistical analysis of the other parameters must be considered exploratory in nature. We carried out two main experiments of 8 and 16 weeks duration, respectively, first to confirm the findings and second to see whether the effect of $1,25(OH)_2D_3$ is more pronounced as times passes. The experiments showed that in $1,25(OH)_2D_3$ treated subtotally nephrectomized rats (SNX) (*i*) the mean glomerular volume tended to be less (the difference was significant in the 8 week experiment) and (*ii*) the glomerulosclerosis index was significantly lower. These findings are in agreement with the working hypothesis that $1,25(OH)_2D_3$ interferes with glomerular growth and scarring. The *in vivo* relevance of this finding is supported by a repeat experiment that showed that eight weeks after SNX the urinary albumin excretion rate was significantly lower in animals treated with $1,25(OH)_2D_3$ compared to pair-fed animals treated with ethanol. The working hypothesis was further supported by ancillary experiments that documented less proliferation of renal cells using the PCNA technique [28]. With respect to the genesis of renal scarring, it is of interest that the expression of the sclerosis-promoting cytokine TGF-$\beta$ in tubular cells and in the vascular wall was markedly less in SNX animals treated with $1,25(OH)_2D_3$ when evaluated by non-radioactive *in situ* hybridization.

We emphasize that we examined a non-hypertensive model of relatively modest renal damage. Further studies are required to assess whether the results can be generalized, that is, whether they can be extrapolated to hypertensive models and models of more severe glomerulosclerosis.

The above significant effect of $1,25(OH)_2D_3$ on glomerular volume after 16 weeks is of interest because increased glomerular volume is apparently a permissive factor for the genesis of

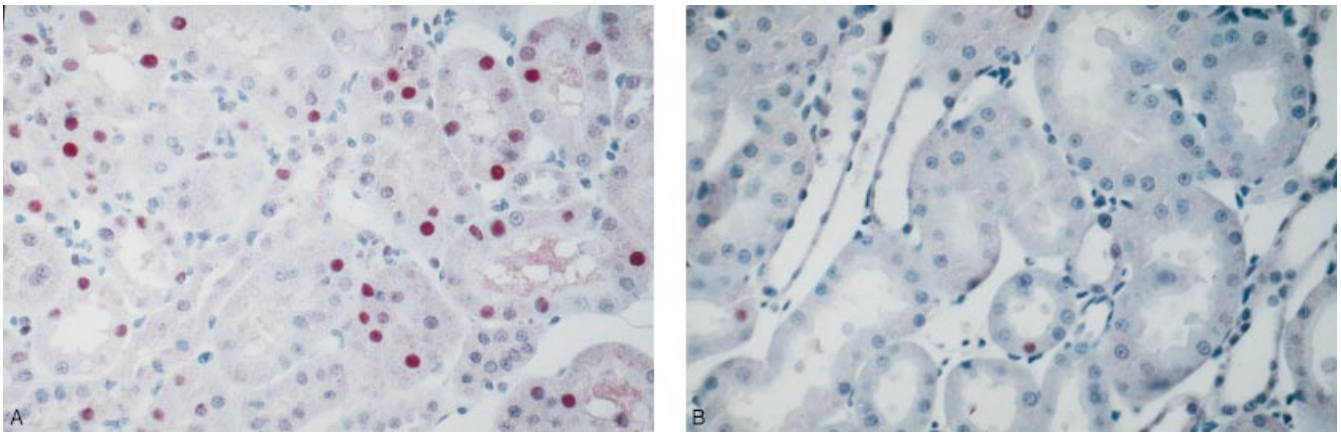


**Fig. 2. PCNA staining in tubules of parathyreoidectomized (PTX), subtotally nephrectomized rats.** Comparison of animal without (*A*) and with (*B*) $1,25(OH)_2D_3$ treatment (magnification ×100).

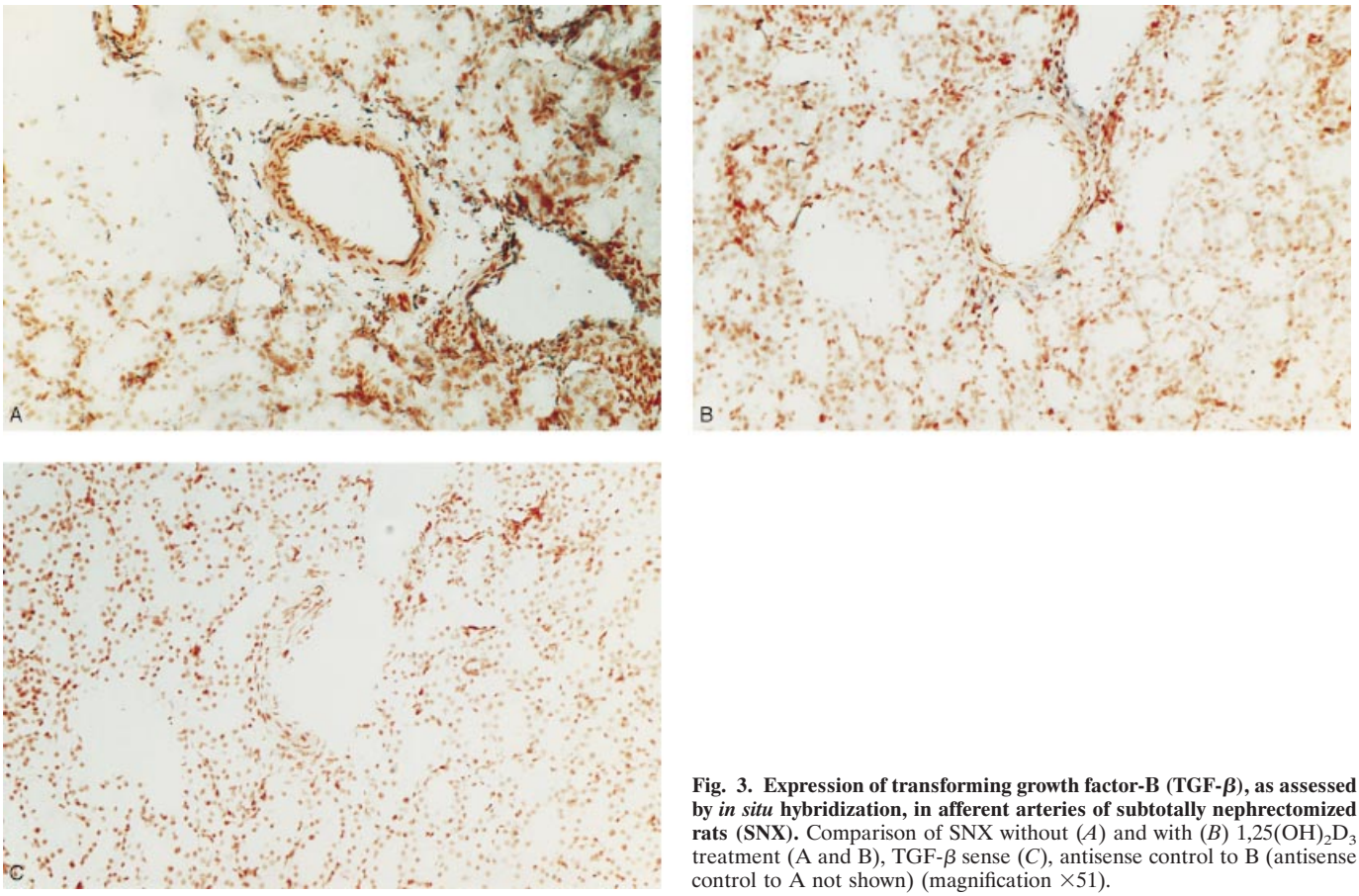


**Fig. 3. Expression of transforming growth factor-B (TGF-β), as assessed by *in situ* hybridization, in afferent arteries of subtotally nephrectomized rats (SNX).** Comparison of SNX without (*A*) and with (*B*) $1,25(OH)_2D_3$ treatment (A and B), TGF-β sense (*C*), antisense control to B (antisense control to A not shown) (magnification ×51).

glomerulosclerosis. This is illustrated by the experiment of Yoshida, Fogo and Ichikawa [19], who found glomerulosclerosis only when renal damage was associated with glomerulomegaly. Stimulation of glomerular growth also apparently contributes to the development of glomerulosclerosis in transgenic animals expressing bovine growth hormone [29]. The mechanism through which increased glomerular volume promotes glomerulosclerosis has not been finally settled. One plausible hypothesis postulates that when glomerular volume increases the domains controlled by individual podocytes increase in parallel [30]. Since podocytes are postmitotic cells, their number cannot increase. Expansion of the filtering surface domain is thought to strain the capacity of podocytes to compensate or adapt, thus leading to local areas of podocyte malfunction [30–33]. An alternative or complementary idea is that capillary wall stress must increase with increasing glomerular volume and capillary diameter [34].

When designing the above study we tried to avoid several known confounding factors. First, the uremic milieu may introduce confounding variables, such as metabolic acidosis, severe hyperphosphatemia, severe hyperparathyroidism, etc. In order to circumvent such problems, we intentionally induced only a moderate reduction of the number of glomeruli so that the animals were not severely uremic. Second, elevated blood pressure is of overriding importance for the development of glomerular injury [35]. Consequently, blood pressure was monitored (and as an ancillary indicator of blood pressure-induced target organ damage we also assessed left ventricular weight). Both indicators showed that the animals were not severely hypertensive and also documented that $1,25(OH)_2D_3$ had no significant effect on blood pressure. Another variable is the type of subtotal nephrectomy. Vascular ligation causes more marked hyperreninism [36] than surgical resection as adopted in the present study [37]. This point is of interest in view of the known effect of an activated renin system on the development of glomerulosclerosis.

In the $1,25(OH)_2D_3$ treated SNX body wt was significantly lower than in ethanol treated SNX in the eight weeks experiment. A significant difference was no longer seen 16 weeks after SNX. Food consumption had not been significantly different, but to further exclude any artifact of food intake we carried out a repeat eight week experiment (Table 4) in which $1,25(OH)_2D_3$ treated SNX were pair-fed with ethanol treated SNX. Despite an identical food intake the final body wt was significantly lower in $1,25(OH)_2D_3$ treated animals. Despite no difference in food intake, albuminuria was significantly greater in the ethanol treated SNX. Consequently, we can safely exclude an artifact resulting from lower intake of protein, sodium or phosphate. We cannot definitely exclude the possibility that body wt *per se* influences development of glomerulosclerosis independent of food intake as described elsewhere [38]. Whether lower body wt in $1,25(OH)_2D_3$ treated animals points to increased metabolism, diminished anabolism, or fluid loss cannot be decided on the basis of these findings.

Based on a pilot experiment and previous studies in this laboratory [39] a dose of $1,25(OH)_2D_3$ was selected that did not cause hypercalcemia. Nevertheless, some increases of serum calcium and urinary calicum excretion were seen. We intentionally used delivery by osmotic minipumps, since previous studies had shown that the effect of $1,25(OH)_2D_3$ depends on the concentration time profile, that is, intermittent versus continuous administration [39]. The dose administered was in the range of estimated daily endogenous synthesis of $1,25(OH)_2D_3$ [40]. The achieved concentration of $1,25(OH)_2D_3$ were not in the frankly pharmacological range.

While in the above study significantly less glomerulosclerosis was documented in animals treated with $1,25(OH)_2D_3$, no significant effect was seen with respect to tubulointerstitial or vascular damage. This negative finding is presumably the result of the very modest degree of renal damage. The vascular and tubulointerstital lesions were so minor that the method may not have been sensitive enough to detect an effect of $1,25(OH)_2D_3$. To confirm or refute this hypothesis rats with more advanced renal failure must be examined.

The kidney is known to have vitamin D receptors (VDR), particularly in tubular epithelial cells [16], and also in glomerular cells [15]. It is conceivable that the effect of $1,25(OH)_2D_3$ is mediated via direct interaction with the vitamin D receptor. To exclude an alternative possibility, that is, that the effect seen with $1,25(OH)_2D_3$ was due to suppression of PTH secretion, we compared cell proliferation, as evaluated by PCNA technique, in parathyroidectomized SNX animals treated with ethanol or $1,25(OH)_2D_3$, respectively. In animals treated with $1,25(OH)_2D_3$ the number of PCNA positive cells, predominantly in the tubulointerstitial space, was significantly less. This finding excludes an artifact from changes in PTH concentration, but it does not provide information whether the effect of $1,25(OH)_2D_3$ is mediated via genomic or non-genomic effects of the secosterole. Whether responsiveness to $1,25(OH)_2D_3$ is altered secondary to defective interaction with the retinoic acid receptor (RXR), as suggested by the experiments of Sawaya et al [41] was not explored in this study.

The outcome of the present study extends previous *in vivo* [14] and *in vitro* [15, 16] studies and shows that an inhibitory effect of $1,25(OH)_2D_3$ on renal cell proliferation can also be seen in the ablation model of renal damage. Whether suppressed cell proliferation and attenuation of glomerular enlargement are the only explanations for this finding is uncertain. Involvement of T-cells and macrophages in the genesis of progression has been documented in numerous studies. In this context, it is of interest that $1,25(OH)_2D_3$ [42] and vitamin D analogs [43] prevent renal injury in immune models of renal damage, that is, active Heyman nephritis and mercury-chloride-induced autoimmune glomerulonephritis of the Brown Norway rat. Diminished recruitment of T and B cells, inhibition of the synthesis of various interleukins (IL), especially IL-2 [44, 45] and IL-6 [46], defective antigen presentation by lowering expression of MHC-II molecules [47, 48] and other mechanisms have been proposed, but not definitely proven. Nevertheless, it is of interest that active vitamin D metabolites also have been shown to interfere with autoimmune damage in non-renal models, such as diabetes mellitus [49], encephalitis [50] and non-renal allograft survival [51].

The results of our study suggest that development of renal fibrosis, at least glomerular fibrosis, is attenuated by $1,25(OH)_2D_3$. In other cell systems $1,25(OH)_2D_3$ has been shown to stimulate synthesis of fibronectin [52], and a recent presliminary short term *in vitro* study using mice proximal tubular cells found stimulation of TGF-$\beta_1$ [53]. This study is not necessarily in conflict with our observation, since in models of renal injury the effect of TGF-$\beta$ varies with time; notably, TGF-$\beta$ has antiproliferative effects (which may predominate in a early phase) and pro-fibrotic effects (which may predominate in a later phase) [54]. It is conceivable that the effect of $1,25(OH)_2D_3$ on TGF-$\beta$ is time dependent, and this possibility requires further study.

### What are the implications of the above findings?

Since the dose of $1,25(OH)_2D_3$ administered was not in the pharmacological range, it is conceivable that reduced renal synthesis of the secosterole $1,25(OH)_2D_3$ and lower concentrations of endogenous $1,25(OH)_2D_3$ play a role in the genesis of glomerulosclerosis and possibly progression of renal failure. This idea becomes even more attractive since the concentrations in the vinicity of the proximal tubular cell, the site of endogenous synthesis of $1,25(OH)_2D_3$, may be considerably higher than in the systemic circulation [16]. Further experiments are necessary to confirm or refute this hypothesis.

**Does the above observation have potential clinical implications?**

In the past the concern about potentially nephrotoxic effects of $1,25(OH)_2D_3$ [2] had already been allayed by the results of controlled clinical studies [3–7], which failed to show accelerated progression in patients on treatment with active vitamin D. Such concerns are thoroughly dispelled by the present experimental study, which even documents a beneficial effect of $1,25(OH)_2D_3$ on glomerular injury. Though significant, the effect is modest in magnitude and would not justify administration of $1,25(OH)_2D_3$ with the aim of reducing progression, even if the above animal data could be extrapolated to human beings. At the very least, however, changes in $1,25(OH)_2D_3$ concentration must be considered as a potential confounder in future experiments on progression. Furthermore, administration of $1,25(OH)_2D_3$ provides an interesting experimental tool to modulate progression.

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