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C-type natriuretic peptide inhibits mesangial cell proliferation and matrix accumulation *in vivo*

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C-type natriuretic peptide inhibits mesangial cell proliferation and matrix accumulation *in vivo*. Local C-type natriuretic peptide (CNP) production and CNP receptor expression have been demonstrated in glomeruli. However, the glomerular (patho-)physiological functions of CNP are largely unknown. We therefore investigated the effects of CNP on mesangial cell proliferation and matrix accumulation in the rat mesangioproliferative anti-Thy 1.1 model. Over seven days rats received a continuous infusion (1 μg/kg/min) of either CNP ($N = 6$), an irrelevant control peptide ($N = 3$) or buffer alone ($N = 6$). Kidney biopsies were performed on days 2, 4 and 8. Few significant differences between the groups were noted on days 2 and 4. Compared to buffer treated rats on day 8, those receiving CNP showed a 35% reduction of glomerular mitoses, a 62% reduction of glomerular uptake of the thymidine analogue BrdU and a significant reduction in glomerular expression of PDGF B-chain. Double immunoperoxidase staining also revealed blunting of proliferating, activated mesangial cells (51% reduction of α-smooth muscle actin-/BrdU-positive cells) and macrophage influx. Moreover, there was a marked reduction of mesangial collagen IV and fibronectin accumulation at the protein and mRNA level. Rats receiving the control peptide were indistinguishable from buffer treated rats. Systemic blood pressure was reduced by 10 to 20% in both CNP and control peptide treated rats on day 8, excluding that the findings were due to hemodynamic effects of CNP. Our findings demonstrate that CNP is involved in the regulation of mesangial cell proliferation and matrix production in vivo. The data suggest the existence of a glomerular natriuretic peptide system that may regulate tissue homeostasis and contribute to resolution of mesangioproliferative diseases.

C-type natriuretic peptide (CNP), a recently identified member of the natriuretic peptide family, was initially isolated from brain [1] and hence thought to be a neurotransmitter. Later studies, however, have detected CNP in human plasma and demonstrated its production, among others, in glomeruli, parts of the renal vasculature (vasa recta bundles and arcuate arteries) as well as in the wall of large extrarenal vessels [2–4]. It was also shown to be expressed by macrophages [5]. CNP exerts biological actions via the natriuretic peptide B-type guanyl cyclase-linked receptor (ANPR-B) and induction of the second messenger cGMP. The expression of ANPR-B transcripts in glomeruli has been described, but low copy numbers prevented a more definitive intraglomerular localization of the receptor [2, 4, 6]. However, *in vitro* ANP B-receptors as well as CNP-inducible cGMP production were demonstrated in glomerular mesangial and epithelial cells [7, 8].

The biological role(s) of CNP in the kidney are not yet fully understood. Unlike the two other natriuretic peptides ANP and BNP, its natriuretic and diuretic effects appear limited and its vasorelaxant action in arterial vessels is significantly lower than that of ANP and BNP [9, 10]. In recent studies, potent antiproliferative effects of CNP on vascular smooth muscle cells have been reported both *in vitro* and *in vivo* [11, 12]. Given the similarities of mesangial cells and vascular smooth muscle cells, the actions of CNP have also been investigated in the former cell type. However, so far no effect of CNP on the growth of cultured human mesangial cells has been detected [8]. Effects of CNP on mesangial cells *in vivo* have not yet been elucidated.

Mesangial cell proliferation and matrix accumulation characterize various progressive human glomerular diseases like IgA nephropathy, membranoproliferative glomerulonephritis, variants of idiopathic focal sclerosis, lupus nephritis and diabetic nephropathy [13, 14]. Various mediators, including platelet-derived factor (PDGF), basic fibroblast growth factor (bFGF; FGF-2), insulin-like growth factor-1 (IGF-1) as well as transforming growth factor-β (TGF-β), have been identified, which induce mesangial cell proliferation and/or matrix production *in vivo* [14, 15]. In contrast, few factors are known, which downregulate these processes (such as heparan sulphate proteoglykans, ANP) [14, 15]. Given the CNP data described above, we asked whether CNP might represent a member of this latter group. To address this question, we examined the effects of exogenous CNP administration on mesangial cell proliferation and matrix accumulation in the rat model of anti-Thy 1.1 mesangioproliferative nephritis. In this model, rats receive a bolus injection of a complement-fixing, anti-mesangial cell (anti-Thy 1.1) antibody. Following an early phase of mesangial dissolution ("mesangiolysis"), overshooting mesangial cell growth and matrix production occur, which give rise to the pathological picture of mesangioproliferative glomerulonephritis [15].

METHODS

Mesangial cell culture experiments

Rat mesangial cells were established in culture, characterized and maintained as described previously [16]. To examine the antiproliferative effect of CNP ($CNP_{1\text{-}22}$; Bachem, Heidelberg, Germany) on the cultured mesangial cells, cells were seeded in 24

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well plates (Nunc, Wiesbaden, Germany) and grown to subconfluency. They were then growth arrested for 72 hours in medium containing 0.5% FCS. After 72 hours, the medium was replaced by fresh medium containing 10% FCS plus various concentrations of CNP. Cell proliferation was determined using [^{3}H]-thymidine (Amersham, Braunschweig, Germany) incorporation rates, 18 hours after addition of the stimulus as described [17].

Experimental design

All animal experiments were approved by the local review board. Male Wistar rats (Charles River, Sulzfeld, Germany) weighing 160 to 180 g at the start of the study were used for all experiments.

Anti-Thy 1.1 mesangial proliferative glomerulonephritis was induced by injection of a monoclonal anti-Thy 1.1 antibody (clone OX7; European Collection of Animal Cell Cultures, Salisbury, UK). Rats received 1 mg OX7 IgG per kg body wt intravenously at the start of the experiment. Twenty-four hours later, microosmotic pumps (model Alzet 2001, Charles River, Sulzfeld, Germany) were implanted subcutaneously and the rats received a continuous infusion of either CNP (1 μg/kg/min = 0.5 nM/kg/min; $N = 6$), an irrelevant control peptide (see below; $N = 3$) or buffer alone (PBS containing 5 mM HCl, 5% D-glucose and 100 mg/ml mannitol; $N = 6$) over seven days. The irrelevant 28 amino acid control peptide (a kind gift of the Niedersächsisches Institut für Peptidforschung, Hannover, Germany) had a molecular weight of 3486 Daltons, comparable to that of CNP (22 amino acids, 2197 Daltons) and a similar amino acid composition. Pilot experiments (kindly performed by Dr. M. Meyer, Niedersächsisches Institut für Peptidforschung) had demonstrated that the loop-structure of CNP, which is based on a disulfide-bond and determines biological activity, remained unchanged when the peptide was stored at 37°C for seven days.

Twenty-four hour urine collections were performed on days 3 and 7 after disease induction. Surgical (maximum 2 per rats) or autoptic renal cortical biopsies were performed on days 2, 4 and 8 after disease induction. Systolic arterial pressure was measured in conscious restrained rats by tail-cuff plethysmography on days −1, 3 and 7. The thymidine analog 5-bromo-2′-deoxyuridine (BrdU; Sigma, Deisenhofen, Germany) was injected intraperitoneally at one hour prior to sacrifice on day 8. Following the renal biopsies on day 8, glomeruli were isolated and glomerular RNA was extracted.

In the kidney biopsies, the number of glomerular mitoses, the degree of mesangiolysis, the frequency of glomerular microaneurysms and the number of apoptotic cells in glomeruli were determined. In addition, immunostaining and *in situ* hybridization were performed to detect glomerular expression of the intermediate filament protein α-smooth muscle actin, infiltrating cells (monocytes/macrophages) and extracellular matrix proteins (type IV collagen, fibronectin).

Renal morphology

Tissue for light microscopy was fixed in methyl Carnoy's solution [18] and embedded in paraffin. Four micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. For each biopsy the number of mitoses in over 30 cross sections (range 30 to 100) of consecutive cortical glomeruli containing more than 20 discrete capillary segments each, was evaluated by one of the authors, who was unaware of the origin of the slides. The percentage of glomeruli with microaneurysms were counted. The degree of mesangiolysis was scored as described previously [16].

Immunoperoxidase staining

Four micrometer sections of methyl Carnoy's fixed biopsy tissue were processed by an indirect immunoperoxidase technique as previously described [18]. Primary antibodies included:

- BU-1, a murine monoclonal antibody against bromo-deoxyuridine [19] containing nuclease in Tris buffered saline (Amersham, Braunschweig, Germany).
- 1A4 (Dako, Glostrup, Denmark), a murine monoclonal IgG_{2a} antibody to an NH_2-terminal synthetic decapeptide of α-smooth muscle actin [20].
- PGF-007 (Mochida Pharmaceutical, Tokyo, Japan) a murine monoclonal antibody to a 25 amino acid peptide located near the COOH-terminus of the human PDGF B-chain (kind gift of Mochida Pharmaceutical) [21].
- ED1 (Camon, Wiesbaden, Germany), a murine monoclonal IgG antibody to a cytoplasmic antigen present in monocytes, macrophages and dendritic cells [22].
- Affinity purified polyclonal goat anti-human/bovine type IV collagen IgG preabsorbed with rat erythrocytes (Biozol, Birmingham, AL, USA).
- An affinity-purified IgG fraction of a polyclonal rabbit anti-rat fibronectin antibody (Chemicon, Temecula, CA, USA).

For all biopsies, negative controls consisted of substitution of the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody or normal rabbit or goat IgG.

For the evaluation of the immunoperoxidase stains, the numbers of BrdU positive cells and ED1 positive cells per glomerular cross section were counted as described above. Glomerular staining for α-smooth muscle actin, PDGF B-chain, fibronectin, and type IV collagen was evaluated using a semiquantitative scoring system and the mean score per biopsy was calculated. Each score reflects mainly changes in the extent rather than intensity of glomerular staining and depends on the percentage of the glomerular tuft area showing positive staining: 0 = absent staining or less than 5% of area stained; I = 5 to 25%; II = 25 to 50%; III = 50 to 75%; IV = >75%. We have previously shown that the above scoring system is not only reproducible among different observers, but that the data obtained are highly correlated with those obtained by computerized morphometry [23, 24].

Immunohistochemical double-staining

Double immunostaining for the identification of the type of proliferating cells was performed as reported previously [24] by first staining the sections for proliferating cells with the bromodeoxyuridine antibody (see above) using an immunoperoxidase procedure. Sections were then incubated with the IgG_1 monoclonal antibody α-SM1 against α-smooth muscle actin using an immunoalkaline phosphatase procedure. Cells were identified as proliferating mesangial cells if they showed positive nuclear staining for BrdU and if the nucleus was completely surrounded by cytoplasm positive for α-smooth muscle actin. Negative controls included omission of either of the primary antibodies, in which case no double-staining was noted.

TUNEL staining for the detection of glomerular cell death

In situ detection of apoptotic cell death was performed using terminal desoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) as described [25]. Briefly, 4 μm sections of formaldehyde fixed tissue were deparaffinized, rehydrated and submitted to microwave antigen retrieval in citric acid buffer. Nuclear proteins were then stripped using proteinase K (Boehringer Mannheim, Germany). Next, the slides were incubated with terminal desoxynucleotidyl transferase (Pharmacia, Freiburg, Germany) and biotin-14-dATP (Gibco BRL, Eggenstein, Germany) for one hour at room temperature. After washing, incorporated biotinylated ATP was detected using the ABC kit (Vector, Burlingame, CA, USA) and diaminobenzidine (DAB) plus nickel. Slides were then counterstained with methyl green and coverslipped. Positive controls included a DNAse control (DNAse 1; Sigma), in which all nuclei exhibited positive staining. For negative controls, biotin-14-ATP was omitted resulting in no nuclear staining.

In situ hybridization for type IV collagen mRNA

In situ hybridization was performed on 4 μm sections of biopsy tissue fixed in buffered 10% formalin utilizing a digoxigenin-labeled anti-sense RNA probe for type IV collagen [26] as described [27]. Detection of the RNA probe was performed with an alkaline phosphatase coupled anti-digoxigenin antibody (Genius Nonradioactive Nucleic Acid Detection Kit; Boehringer-Mannheim) with subsequent color development. Controls consisted of hybridization with a sense probe to matched serial sections, by hybridization of the anti-sense probe to tissue sections that had been incubated with RNAse A before hybridization, or by deletion of the probe, antibody or color solution [27]. Glomerular mRNA expression was semiquantitatively assessed using the scoring system described above.

Northern blots

Glomeruli were isolated by differential sieving [28]. All glomerular isolates were checked microscopically and exhibited a purity of greater than 98%. Total RNA was extracted from glomeruli with guanidinium isothiocyanate and subsequent ultracentrifugation through caesium chloride using standard procedures [29, 30]. The RNA content of the samples obtained was determined by UV spectrophotometry at 260 and 280 nm. Samples with an OD 260/280 nm ratio of <1.8 were discarded. Northern analysis for the glomerular expression of α_1(IV) collagen mRNA was performed using digoxigenin labeled riboprobes and the Digoxigenin Nucleic Acid Detection Kit (Boehringer) as described previously [26]. For the detection of PDGF B-chain mRNA, a 326 bp *Eco*RI/*Bam*HI fragment of murine PDGF B cDNA [27] was labeled with ^{32}P using random primers. Ten micrograms of total glomerular RNA were then fractionated by electrophoresis in a 1% agarose gel containing formaldehyde and transferred to a Nylon membrane (Amersham, Braunschweig, Germany). Hybridization was carried out in 520 mM sodium phosphate (pH 7.2), 7% SDS, 1 mM EDTA and 0.15 mM BSA at 60°C for 16 hours. After hybridization, the filter was washed with 40 mM sodium phosphate (pH 7.2), 1 mM EDTA and 1% SDS at 60°C for 30 minutes. It was then exposed to a Kodak X-ray film at −80°C in the presence of an intensifying screen. Stripping of the hybridization signal between the hybridization steps with the PDGF-B probe and a probe for a housekeeping gene (1.2 kb *Pst*I fragment of rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA) was performed by boiling the filter in 0.1% SDS for four minutes. Blots were evaluated by densitometry as described [26].

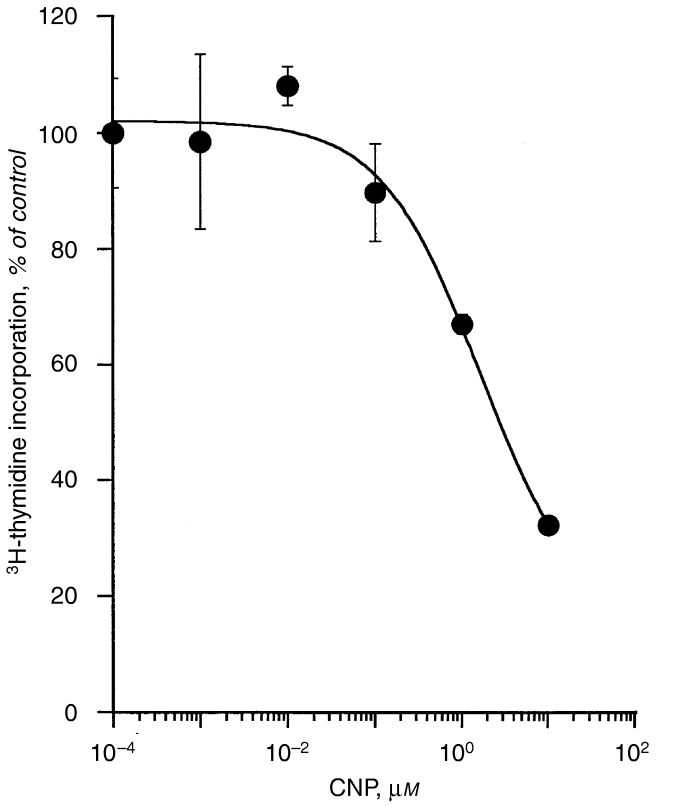

Fig. 1. Effect of C-type natriuretic peptide (CNP) on the growth of cultured rat mesangial cells. Dose-response relationship of CNP-induced inhibition of [^{3}H]-thymidine incorporation. Values are expressed as percentage of [^{3}H]-thymidine incorporation in mesangial cells not exposed to CNP.

Statistical analysis

All values are expressed as mean ± SD. Statistical significance (defined as $P < 0.05$) was evaluated using ANOVA and Bonferroni *t*-tests.

RESULTS

C-type natriuretic peptide inhibits rat mesangial cell proliferation *in vitro*

C-type natriuretic peptide (CNP) inhibited [^{3}H]-thymidine incorporation in a concentration-dependent manner after 18 hours of incubation (Fig. 1). [^{3}H]-thymidine incorporation into the rat mesangial cells was decreased to 70% of controls by 10 μM CNP. Similar reduction rates were also noted in the MTT assay, which detects total numbers of viable cells, where 10 μM CNP led to a 61% reduction of the extinction. Trypan blue dye exclusion was found to be > 95% for both control and CNP treated cells, suggesting that the reduction of [^{3}H]-thymidine incorporation was not due to a cytotoxic effect of the CNP.

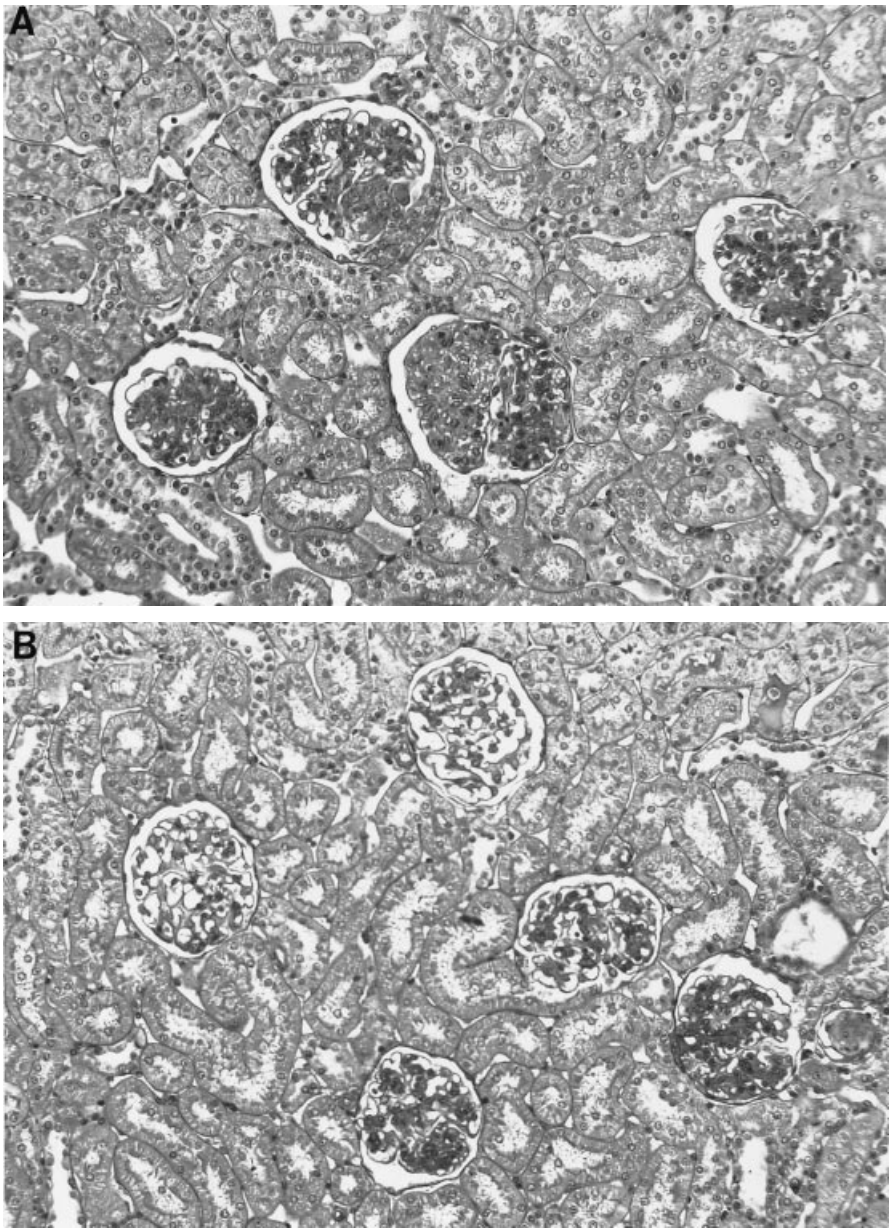

Fig. 2. PAS-stained renal sections obtained on day 8 after induction of anti-Thy 1.1 nephritis from a rat infused with buffer only (*A*) or C-type natriuretic peptide (CNP) (*B*). Compared to buffer infused rats, the CNP infused rat exhibits less mesangial hypercellularity and matrix expansion. Control peptide infused rats were indistinguishable from buffer infused rats (magnification ×200).

C-type natriuretic peptide in rat mesangioproliferative nephritis

Following the injection of anti-Thy 1.1 antibody, buffer treated animals developed the typical course of the nephritis, which is characterized by early mesangiolysis on days 2 and, to a lesser degree, day 4, and followed by a phase of mesangial cell proliferation and matrix accumulation on days 4 and, more prominently, on day 8 [15]. Analysis of PAS-stained renal sections showed that CNP treatment, in comparison to buffer or control peptide infused rats, led to a reduction of mesangioproliferative changes on day 8 with little detectable effect on days 2 and 4 (Fig. 2).

C-type natriuretic peptide reduces glomerular mesangial cell proliferation *in vivo*

Glomerular cell proliferation, as assessed by counting the number of glomerular mitoses, was not different between the three experimental groups on days 2 and 4 after disease induction (Fig. 3). On day 8, the number of mitoses per 100 glomeruli was reduced by 35% in the CNP group as compared to the control peptide and buffer group (Fig. 3). The CNP treated group also displayed a significant reduction of glomerular BrdU positive nuclei on day 8. These latter were reduced to 61% and 51% of the control peptide and buffer group, respectively (Fig. 3). To investigate whether the reduced cell proliferation in CNP treated animals resulted from increased glomerular cell death/apoptosis, we performed TUNEL-staining on the tissue sections of day 8. TUNEL-positive nuclei ranged from 5 to 10 per 100 glomerular cross sections. Counts were not significantly different between the three experimental groups (data not shown).

To specifically assess the effect of CNP on mesangial cells, we immunostained the renal sections for α-smooth muscle actin, which was expressed only by activated mesangial cells [28]. Again,

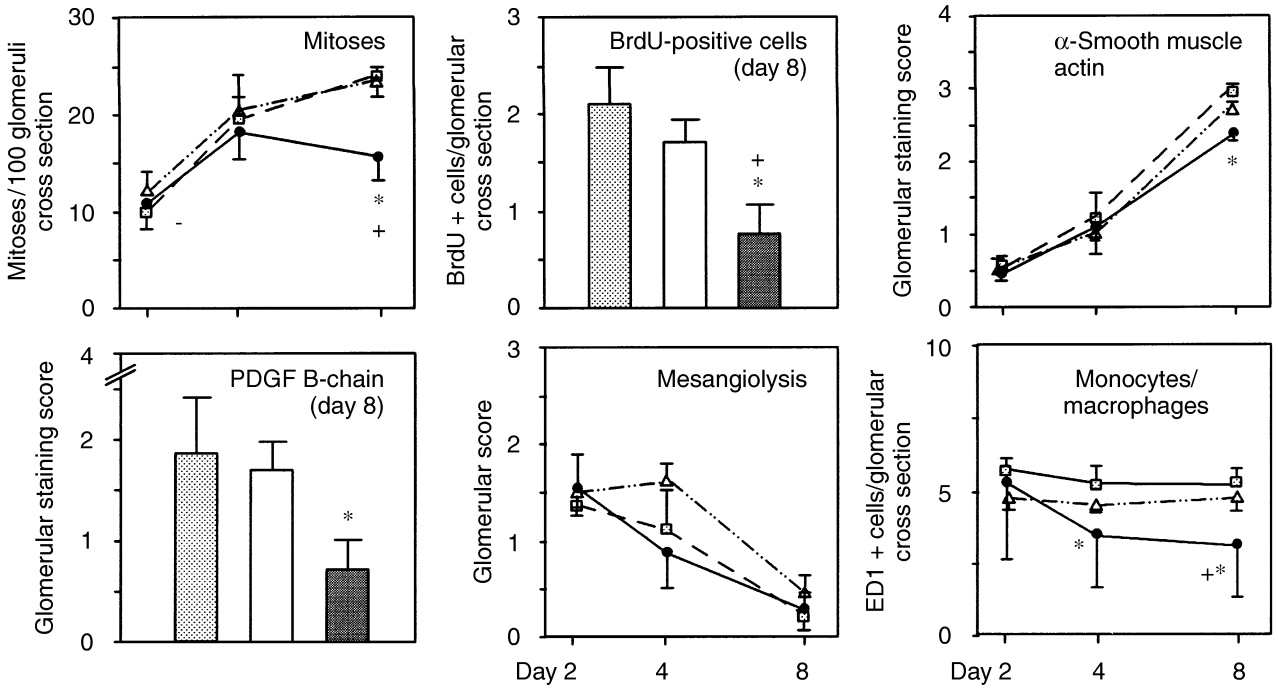

Fig. 3. Effects of C-type natriuretic peptide (CNP) on glomerular proliferation (as assessed by the number of glomerular mitoses and BrdU positive nuclei), mesangial cell activation (as assessed by glomerular *de novo* expression of α-smooth muscle actin), expression of platelet-derived growth factor (PDGF) B-chain, mesangiolysis, microaneurysm formation, and monocyte/macrophage influx in rats with anti-Thy 1.1 nephritis. Symbols are: (▨) buffer infused rats, $N = 6$; (■ and ●) CNP infused rats, $N = 6$; (□ and △, control peptide infused rats, $N = 3$; $^{*}P < 0.05$ vs. buffer infused rats; $^{+}P < 0.05$ vs. control peptide infused rats.

there were no significant differences between the three groups on days 2 and 4. However, the immunostaining scores of α-smooth muscle actin were significantly reduced on day 8 in the CNP treated group (Fig. 3). The effect of CNP on glomerular α-smooth muscle actin expression was most pronounced when glomeruli with more than 50% of the tuft stained (immunohistochemical staining scores III and IV) were analyzed separately. The percentage of such glomeruli was reduced to 52 ± 9% in CNP infused rats as opposed to 83 ± 8% (buffer-infused rats; $P < 0.001$ vs. CNP) and 71 ± 5% (control peptide-infused rats; $P < 0.05$ vs. CNP).

By double immunostaining for BrdU- and α-smooth muscle actin-positive cells on day 8, we specifically assessed the effect of CNP on proliferating, activated mesangial cells. The data showed that CNP reduced the population of BrdU-/α-smooth muscle actin-positive cells by 51%: buffer infused rats 0.57 ± 0.14 cells per glomerular cross sections versus 0.28 ± 0.05 cells in CNP infused rats; $P < 0.05$ (total numbers of BrdU positive cells per glomerular cross section in these studies were 1.09 ± 0.10 in buffer infused rats vs. 0.55 ± 0.13 in CNP infused rats; the lower number of BrdU positive cells in these studies as compared to Fig. 3 relates to differences in staining conditions).

To investigate whether CNP reduces glomerular mesangial cell proliferation and activation indirectly, we investigated the glomerular expression of PDGF B-chain, a central mediator of mesangial cell proliferation, which is overexpressed in anti-Thy 1.1 nephritis [27, 31]. By immunohistochemistry, there was a diminished glomerular protein expression of PDGF B-chain in CNP treated animals (Fig. 3). In contrast, Northern blotting revealed no reduction in the glomerular mRNA expression of PDGF B-chain.

Table 1. Northern blot analysis of whole glomerular RNA for the expression of type IV collagen mRNA and PDGF B-chain mRNA in CNP, control peptide or buffer infused rats on day 8 after disease induction

	CNP infusion	Control peptide infusion	Buffer infusion
Collagen IV mRNA	72%	85%	100%
	72%	95%	100%
PDGF B-chain mRNA	150%	109%	100%
	144%	130%	100%

Results of two separate experiments are presented. Data are expressed as percentage of the optical density observed in buffer infused rats and corrected for the expression of a housekeeping gene (GAPDH).

Rather, CNP treated rats displayed an increase in mRNA to 147% of the buffer group (Table 1).

Reduction of glomerular mesangial cell proliferation did not result in prolongation of mesangiolysis and increased microaneurysm formation in the CNP treated rats as compared to the controls (Fig. 3). Proteinuria was low on both day 3 and 7 after disease induction and was not statistically different between the three groups of rats (day 3, buffer 13 ± 6, control peptide 8 ± 1, CNP 9 ± 5 mg/24 hr; day 7, buffer 18 ± 10, control peptide 21 ± 10, CNP 9 ± 3 mg/24 hr).

C-type natriuretic peptide reduces glomerular cell infiltration *in vivo*

Monocyte/macrophage influx, as detected by the ED-1 antibody, was significantly reduced in the CNP rats on days 4 and 8 after disease induction, but not on day 2 (Fig. 3).

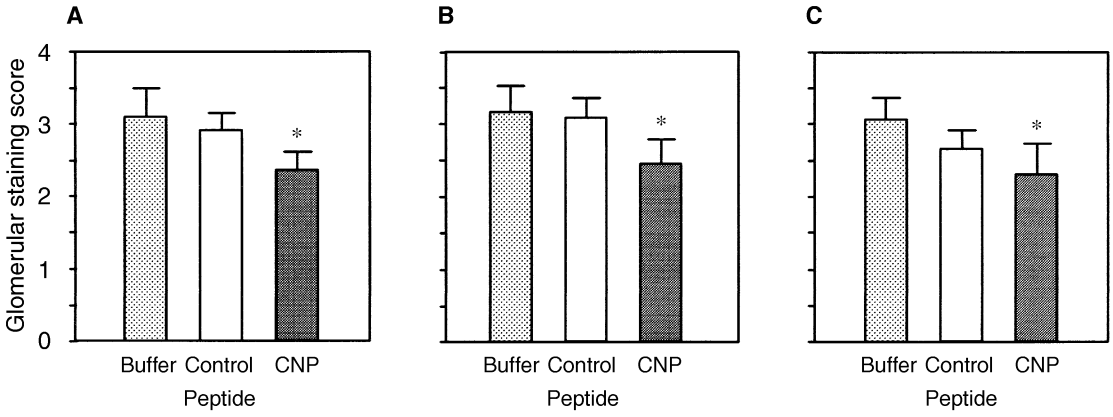

Fig. 4. Effects of C-type natriuretic peptide (CNP) on glomerular accumulation of type IV collagen (immunohistochemistry and *in situ* hybridization) and fibronectin (immunohistochemistry) in rats with anti-Thy 1.1 nephritis on day 8 after disease induction. (*A*) Fibronectin protein. (*B*) Collagen IV protein. (*C*) Collagen IV mRNA. The number of rats in each experiments was: buffer, $N = 6$; control peptide, $N = 3$; CNP, $N = 6$. $^*P < 0.05$ versus buffer infused rats.

C-type natriuretic peptide reduces mesangial matrix accumulation *in vivo*

The glomerular immunostaining scores for the matrix components type IV collagen and fibronectin were significantly reduced in the CNP group as compared to buffer treated rats on day 8 after disease induction (Fig. 4). Similarly, *in situ* hybridization for type IV collagen mRNA also confirmed a significant reduction of type IV collagen production on day 8 (Figs. 4 and 5). By Northern blot analysis the glomerular mRNA expression of collagen IV was decreased in the CNP treated rats to 72% of the buffer group (Table 1).

Hemodynamic effects of C-type natriuretic peptide

As shown in Figure 6, systolic arterial pressure was significantly reduced in CNP treated rats as compared to buffer rats on day 7. However, a similar reduction in blood pressure was also noted in the group that had received the control peptide.

DISCUSSION

In the present study, we demonstrate that a systemic C-type natriuretic peptide (CNP) infusion exerts various beneficial effects on the course of an experimental mesangioproliferative glomerulonephritis. In designing such *in vivo* studies, a central concern is that CNP might interfere with the disease induction itself, particularly with the glomerular binding of the nephritogenic antibody. In order to avoid such problems, we initiated the CNP treatment at 24 hours after injection of the anti-mesangial cell antibody. In previous studies, it has been demonstrated that anti-Thy 1.1 antibody binding to mesangial cells is maximal at one hour [18, 32]. Therefore, neither antibody binding nor complement activation should be affected by the CNP infusion. In support of this assumption, the degree of mesangiolysis, early proteinuria and the early (day 2) glomerular influx of monocytes/macrophages was not affected by CNP or the control peptide.

The first major finding of the present study was that CNP reduces glomerular cell proliferation in anti-Thy 1.1 nephritis. In this model, glomerular cell proliferation is largely due to proliferation of mesangial cells, particularly in the late stages such as day 8 after disease induction [18, 28]. Increased proliferation of glomerular endothelial cells as well as some monocyte/macrophage proliferation have also been detected, but are mostly confined to early phases of the disease [18, 33], that is, day 2 after disease induction, when CNP had no effect on proliferation. This suggests that the effect of CNP was almost exclusively on proliferating mesangial cells. Further support for this hypothesis is derived from the observation that the glomerular expression of α-smooth muscle actin, which is exclusively produced by activated mesangial cells [28], was reduced by CNP. In addition, our data suggest that the reduction of glomerular hypercellularity in CNP infused rats was due to reduced cell proliferation rather than to increased cell removal via apoptosis, which is the major pathway of resolution in this disease [25].

One of the central mitogens for mesangial cells is PDGF B-chain [31]. In cell culture, induction of PDGF B-chain appears to be an auto-/paracrine common final pathway, by which many growth factors induce mesangial proliferation [34]. It was therefore of interest to investigate whether CNP might exert its antiproliferative effect via a down-regulation of mesangial PDGF B-chain production. While the immunohistochemical analysis indeed demonstrated reduced expression of PDGF B-chain in CNP treated rats, the expression of glomerular PDGF B-chain mRNA increased mildly in the CNP group. Since glomerular PDGF B-chain mRNA was normalized via GAPDH mRNA expression, these data suggest that glomerular PDGF gene transcription on a single cell level had increased in CNP infused rats. It is possible that a concomitant increase in protein expression was not detected by immunohistochemistry, since the scoring system is targeted to evaluate the extent of staining (that is, indirectly the number of positive cells) rather than the intensity of cellular staining. This suggests that CNP led to a reduction of mesangial cell proliferation independent of PDGF, which in fact may have increased on a single cell level. Alternatively, however, our *in vivo* data do not allow us to fully exclude that CNP might have induced a post-transcriptional block in the PDGF synthesis and thereby exerted some of its antiproliferative action via decreased translation of PDGF.

A second major finding was that CNP also reduced the glomerular monocyte/macrophage counts in the late stages of the

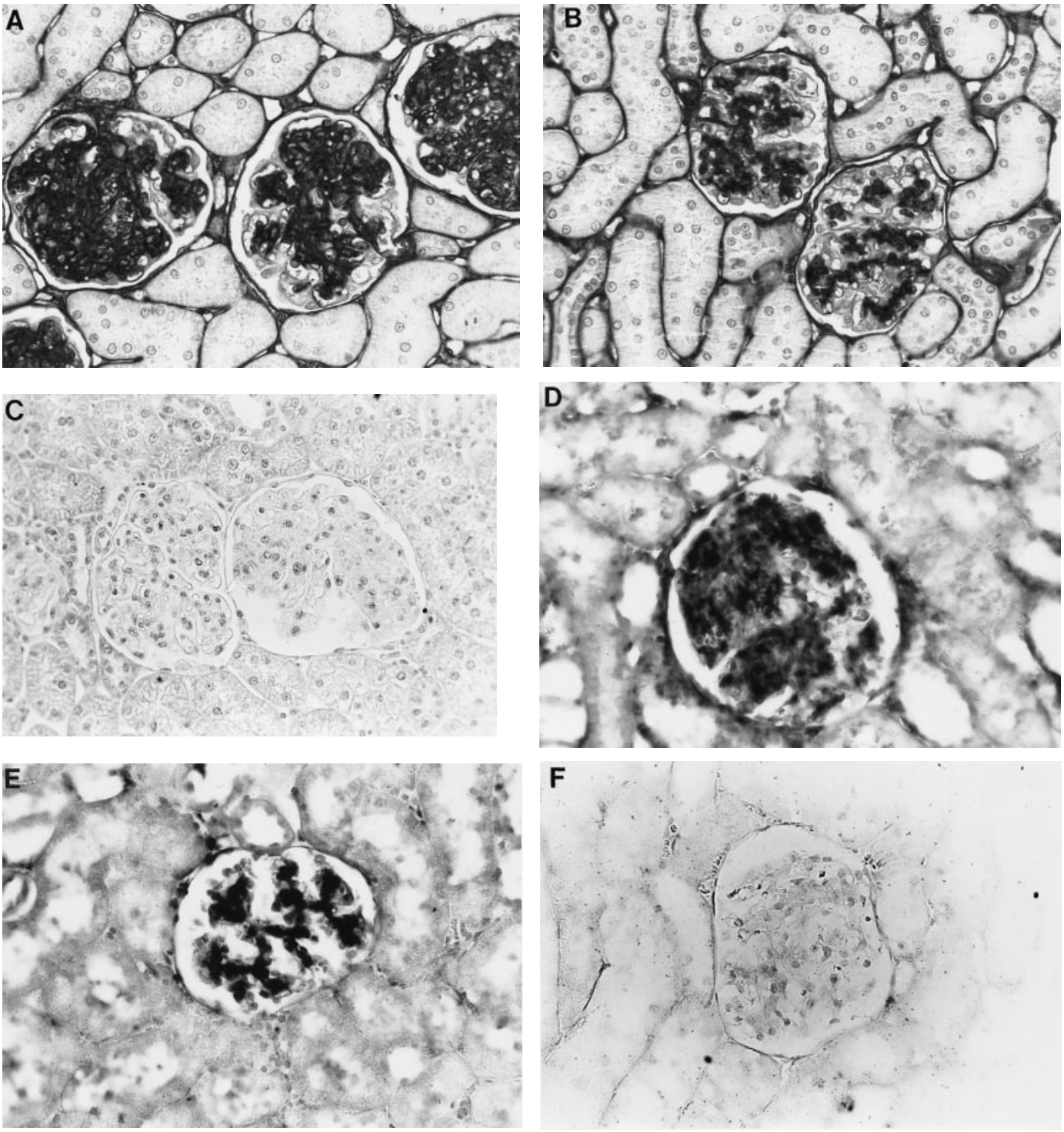

Fig. 5. Glomerular type IV collagen production as assessed by immunohistochemistry (*A, B, C*) and *in situ* hybridization (*D, E, F*) in rats with anti-Thy 1.1 nephritis on day 8 after disease induction. While heavy collagen IV protein and mRNA overexpression is noted in the buffer treated animal (A, D), both collagen IV protein and mRNA expression is markedly reduced in the CNP infused rats (B, E). (C) No specific staining is noted in a section of a rat with anti-Thy 1.1 nephritis when the antibody against type IV collagen is replaced by irrelevant goat IgG. (F) No specific hybridization signal is noted in a section of a rat with anti-Thy 1.1 nephritis when a sense probe for type IV collagen is used for *in situ* hybridization (magnification ×400).

disease. Glomerular macrophage influx in the anti-Thy 1.1 nephritis model has been shown to depend to a large degree on the mesangial production of monocyte chemoattractant protein-1 (MCP-1) [35]. Further, *in vitro* studies are therefore warranted to examine whether CNP can reduce the mesangial cell production of chemotactic proteins.

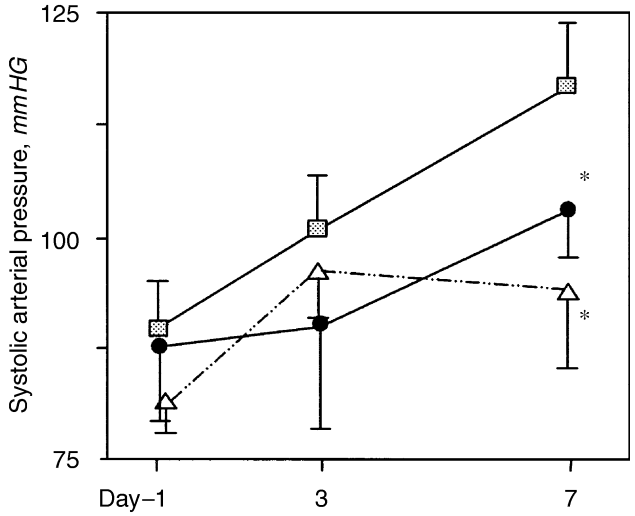

Fig. 6. Systolic arterial pressure measurements on days −1, 3 and 7 after induction of anti-Thy 1.1 nephritis in rats receiving a buffer infusion ($N = 6$; ▨), control peptide ($N = 3$; △) or CNP ($N = 6$; ●). $^*P < 0.05$ versus buffer infused rats.

Third, the current study shows that CNP reduced glomerular matrix accumulation. We and others have previously demonstrated that nearly all mesangial matrix proteins are overproduced in the anti-Thy 1.1 model [36, 37]. Immunohistochemical matrix protein deposition in the nephritis was reduced by antagonism of TGF-β [38] and PDGF [39] as well as by less specific inhibitors of mesangial cell growth, such as heparins [16, 40, 41]. The present study extends these findings by showing that CNP, similar to decorin [38], may represent an endogenous down-regulator of glomerular extracellular matrix synthesis (whether this effect is a direct one of CNP on matrix synthesis or is an indirect consequence of the antiproliferative activity of CNP remains to be determined). Besides glomerular proteases [42], CNP may therefore play an important role in the resolution of glomerular mesangioproliferative changes.

Apart from the glomerular effects of CNP, we also noted a significant reduction of systemic blood pressure in CNP-infused rats. Furthermore, others have shown that CNP, at least in the split hydronephrotic kidney, induces vasodilation of both pre- and postglomerular vessels [43]. Conceivably, reduction of glomerular blood pressure might ameliorate the mesangiolytic changes and thereby reduce the subsequent need for mesangial cell proliferation and matrix production. Several findings argue against this possibility, however, and support our notion that the observed effects of CNP on mesangial cells *in vivo* did represent direct effects: (*1*) infusion of the control peptide had a similar hypotensive potency, yet did not affect mesangioproliferative changes; (*2*) it has previously been shown that a hydralazine-induced 20% reduction of systemic blood pressure in rats with anti-Thy 1.1 nephritis affected neither glomerular cell proliferation, macrophage influx, nor matrix deposition [44].

In summary, our data identify exogenous CNP as a potent regulator of mesangial cell proliferation, matrix production and, potentially, chemoattractant production *in vivo*. Data obtained in cultured bovine carotid endothelial cells show that in endothelial cells the production and release of CNP is stimulated by TGF-β, FGF-2 and inflammatory cytokines such as tumor necrosis factor-α and interleukin-1 [3]. Given that CNP is produced in glomeruli [2–4] and that many of the aforementioned cytokines are (over-)expressed in anti-Thy 1.1 nephritis [15, 36], it is tempting to speculate that endogenous, locally or systemically produced CNP might be involved in the resolution phase of the nephritis. If this role of a glomerular natriuretic peptide system, analogous to that of the postulated vascular natriuretic peptide system [45], can be established through the usage of specific CNP-antagonists, both the exogenous CNP administration and the amplification of endogenous CNP production might become interesting therapeutic approaches to mesangioproliferative diseases.

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