D-Penicillamine reduces renal injury in the remnant model of chronic renal failure in the rat

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Abstract

Background. Glomerulosclerosis and interstitial fibrosis, which are cardinal features of the end-stage kidney, result from accumulation of extracellular matrix proteins, particularly collagen, in the glomerular mesangium and renal interstitium. This study examined the effect of D-penicillamine (DPC), which inhibits collagen deposition, on disease progression in the remnant kidney.

Methods. Two groups of 10 rats underwent two-thirds nephrectomy and were pair-fed 20% casein paste (Gp 1) or the same paste supplemented with 90 mg/kg body wt per day of DPC (Gp 2). Two further groups of five non-nephrectomized animals also received 20% casein paste either alone (Gp 3) or supplemented with DPC (Gp 4). In a further experiment, systolic blood pressure was compared at 1 and 4 weeks after nephrectomy in eight DPC-treated remnants and eight untreated controls.

Results. Gp 2 developed significantly less proteinuria than Gp 1 (41 ± 9 vs 142 ± 33 mg/24 h at 6 weeks, \( P < 0.005 \); 136 ± 36 vs 282 ± 59 mg/24 h at 12 weeks, \( P < 0.05 \)). At sacrifice after 12 weeks, glomerular filtration rates were higher (1.34 ± 0.08 vs 1.07 ± 0.1 ml/min, \( P < 0.05 \)), kidney total collagen content was lower (14.9 ± 1.5 vs 26.9 ± 5.4 mg/kidney, \( P < 0.05 \)) and glomerular abnormalities, interstitial fibrosis and lymphocytic infiltration were less marked in Gp 2 compared with Gp 1. DPC had no effect on protein excretion, total kidney collagen or GFR in non-nephrectomized rats, and did not influence the early rise in blood pressure seen after two-thirds nephrectomy.

Conclusions. These findings demonstrate that DPC reduces renal injury in the remnant kidney, and raise the possibility of a therapeutic role for DPC in the treatment of patients with chronic renal failure.

Key words: collagen; D-penicillamine; fibrosis; remnant kidney

Introduction

The histological appearance of the end-stage kidney is characterized by diffuse fibrosis, involving both glomerular and interstitial compartments, irrespective of the underlying cause of renal disease. The normal mesangial matrix comprises types IV collagen, laminin and heparan sulphate [1]. In glomerulosclerosis, the deposition of these proteins is increased, and collagen types I and III can also be identified. In the interstitium, the most abundant matrix proteins are collagens types I and III [2], and in experimental models of chronic renal disease the deposition of these proteins increases as a result of both increased production and decreased degradation [2–4]. In addition, collagen type IV, which is normally not present in the renal interstitium [5], has been identified in rats with PAN nephrosis [2] and obstructive nephropathy [4]. Since glomerulosclerosis and interstitial fibrosis are central to the progression of chronic renal injury, and collagens are the major ECM proteins responsible for fibrosis, manoeuvres that reduce collagen deposition may delay the progression of chronic renal failure.

D-Penicillamine (DPC) has been shown to inhibit the deposition of mature collagen in the skin [6] and kidneys [7] of patients with scleroderma, a condition characterized by cutaneous and visceral fibrosis. The effects of DPC in other forms of chronic renal injury and in experimental renal disease have not, however, been examined. The aim of this study was therefore to assess the ability of DPC to influence collagen deposition and disease progression in an animal model of chronic renal failure, the two-thirds nephrectomized rat.

Methods

Animal protocols

Two groups of 10 female Wistar rats (initial weight \( \approx 230 \) g) underwent right nephrectomy and ligation of one branch of the left renal artery as a single procedure under general anaesthesia (2.7 ml/kg of Hypnorm/Hypnovel/water 1:1:2 v/v intraperitoneally). Post-operatively, the animals were
housed in individual cages with constant temperature and humidity, and a 12-h light–dark cycle. The untreated group (Gp 1) received a powdered diet (ICN no. 960259), containing 20% casein (w/w), offered as a paste mixed with water (1:1 w/w) and methylcellulose (2 g/100 g). The treated group (Gp 2) were pair-fed the same feed supplemented with D-penicillamine (Sigma, UK) in a dose calculated to provide 90 mg drug/kg body weight per day. To establish any effect of treatment with D-penicillamine on normal kidneys, two groups of five non-nephrectomized animals (Gps 3 and 4) were pair-fed the same feed supplemented in Gp 4 with D-penicillamine. On the 6th and 12th post-operative week, the animals were placed in metabolic cages for collection of 24-h urine samples. After 12 weeks, animals were weighed, and a 10-μCi dose of 51chromium-labelled ethylenediaminetra-acetic acid (EDTA) was injected into a tail vein. At 60 min later, animals were sacrificed by exsanguination under general anaesthesia (2.7 ml/kg of Hypnorm/Hypnovel/water 1:1:2 v/v i.p.). A 50-μl aliquot of plasma was counted using a Cobra autogamma 5002 counter (Canberra Packard, Pangbourne, UK), and the GFR was calculated using an estimated volume of distribution for [51Cr]EDTA according to the method of Layzell and Miller [8]. Serum was analysed for urea and creatinine, and kidneys were removed, weighed and divided into two by sectioning coronally. One portion was fixed in formol saline for histological examination, and the other was retained for estimation of kidney collagen content.

To assess whether treatment with DPC had a direct antihypertensive effect in two-thirds nephrectomized rats, two further groups of eight female Wistar rats underwent right nephrectomy and partial infarction of the contralateral kidney as described above. Post-operatively, the first group received standard 20% casein feed, whilst the second group were pair-fed the same feed supplemented with DPC in a dose calculated to provide 90 mg drug/kg body weight per day as before. Blood pressure was measured in each animal after 1 and 4 weeks using a Harvard tail cuff sphygmomanometer connected to a Coupler FC 100 chart recorder.

### Collagen assay

Total kidney collagen content was determined using a hydroxyproline assay. The half kidney retained for collagen analysis was divided horizontally through the mid-pole to produce two equal pieces of tissue containing renal cortex, medulla and pelvis. One of these pieces was used for collagen analysis, and was weighed, cut into small (approximately 1 mm3) pieces, then reduced to a pulp in a ground-glass organ grinder. The resulting tissue was hydrolysed in 6N HCl at 110°C for 16 h, evaporated to dryness, suspended in deionized water, lyophilized, and resuspended in 2 ml of buffer. The hydroxyproline concentration of this solution was determined by the method of Stegemann and Stalder [9]. A 1-ml aliquot of the sample was mixed with 0.5 ml of a 0.05-M solution of Chloramine T, then left at room temperature for 20 min to oxidize. After oxidation, 1 ml of a 1.2-M solution of Ehrlich’s aldehyde reagent (dimethylaminobenzaldehyde) and perchloric acid (30%) was added, and the resulting solution was incubated at 60°C for 15 min. After cooling, absorbance of the solution was determined at 550 nm using a Cecil CE 2040 spectrophotometer, and hydroxyproline concentration was then calculated by reference to a standard curve. Collagen content of the hydrolysate was estimated by multiplying its hydroxyproline content by 7.42 [3], and total kidney collagen was derived in each case from the tissue collagen concentration and original kidney weight.

### Histology

Formalin-fixed sections were stained by haematoxylin and eosin (H&E), and examined by a histopathologist (PNF) blinded to treatment groups. From each section, 100 glomeruli were sequentially assessed by systematically examining fields using a ×40 objective, moving from capsule to corticomedullary junction in a raster pattern. The vicinity of any infarcted areas was avoided. The number of glomeruli showing obvious abnormalities (i.e. fibrinoid necrosis or severe sclerosis) at this magnification was counted. Subsequently the section was reviewed overall and each of the following features was subjectively graded on a scale of 0 (absent) to 2: Interstitial lymphocytic infiltration (1 = infiltrates evident only on high magnification; 2 = groups obvious on low power).

- Interstitial fibrosis (1 = focal; 2 = widespread).
- Tubular atrophy (1 = occasional atrophic tubules; 2 = areas of atrophy obvious on low power).
- Tubular casts (1 = scattered separate tubular casts; 2 = groups).

Gomerular size on the H&E sections was measured by image capture using a JVC TK-1280E video camera and the frame grabber board of an Apple Macintosh 7100/80AV computer. The image analysis program NIH-Image was used (courtesy of its author Wayne Rasband and the National Institutes of Health of the USA). Ten glomeruli from each section were counted; no allowance was made for variation in apparent size due to non-polar sectioning on the assumption that this would introduce equal error in all the groups. Pixel based measurements were converted to microns by the use of a calibration slide (Graticules Ltd, Tonbridge, Kent).

### Biochemical analyses

Blood urea was measured by urease reagent and serum creatinine by the Jaffe reaction using a Vitatron SPS analyser. Total protein in urine was measured by a dye-binding assay using pyrogallol red (Randox laboratories, UK).

### Statistics

Values are expressed as mean ± standard error. Proteinuria, GFR, urea, creatinine, and total kidney collagen content in treated animals and controls were compared using unpaired t-test. In certain cases, data from groups 1–4 were compared using analysis of variance, and these are indicated in the text.

### Results

The pair-feeding protocol ensured that animals in Gps 1–4 had similar food intake throughout the experiment (overall daily dry food consumption: Gp 1, 32 ± 0.5 g/day; Gp 2, 31 ± 0.4 g/day; Gp 3, 32 ± 0.7 g/day; Gp 4, 32 ± 0.7 g/day, NS by ANOVA). Mean daily penicillamine dose was 94 ± 1.3 mg/kg body wt in Gp 2 and 89 ± 2.3 mg/kg body wt in Gp 4 (NS). Nephrectomized animals all developed proteinuria, but at both 6 and 12 weeks this was signific-
higher in Gps 1 and 2 than in Gps 3 and 4 ($P<0.001$ by ANOVA). In untreated animals (Gps 1 and 3), the collagen content of remnant kidneys was greater than that of controls (Gp 1 vs Gp 3: $26.9\pm 5.4$ vs $10.7\pm 0.8$ mg collagen/kidney, $P<0.05$). DPC treatment had no significant effect on kidney collagen in non-nephrectomized animals, but in the nephrectomized groups, collagen was significantly reduced by DPC (Fig. 3). In addition, kidney weights were significantly lower in Gp 2 than in Gp 1 ($1.42\pm 0.05$ vs $1.7\pm 0.1$ g, $P<0.05$), but were higher in both groups of remnant kidneys than in the corresponding non-nephrectomized group (Gp 1 vs Gp 3: $1.7\pm 0.1$ vs $0.92\pm 0.02$ g, $P<0.001$; Gp 2 vs Gp 4: $1.42\pm 0.05$ vs $1.0\pm 0.03$ g, $P<0.001$). Although kidney collagen per unit kidney weight was lower in Gp 2 than Gp 1 ($10.4\pm 1.0$ vs $15.4\pm 3.3$ mg collagen/g kidney) this difference did not achieve statistical significance.

Histological examination demonstrated no light microscopic abnormalities in kidneys from Gps 3 and 4. All remnant kidneys showed mild chronic renal injury, but abnormalities were more severe in the untreated group. Fibrinoid necrosis or severe sclerosis were seen in $2.75\pm 1.00\%$ of glomeruli from untreated animals compared with $0.38\pm 0.26\%$ of glomeruli in the DPC treated group ($P<0.05$). Untreated animals had significantly higher scores for lymphocytic infiltration and interstitial fibrosis than DPC-treated animals (Table 1). Scores for tubular dilatation and cast formation were also higher in the untreated group, although this effect did not achieve statistical significance. Mean glomerular cross-sectional area was greater in remnant kidneys than controls (overall means $11470\pm 260$ vs $6070\pm 80$ $\mu m^2$, $P<0.0001$), but there was no significant difference in area between Gps 1 and 2 ($11750\pm 390$ vs $11260\pm 400$ $\mu m^2$) and Gps 3 and 4 ($6240\pm 350$ vs $6220\pm 220$ $\mu m^2$).

In the two separate groups of animals whose blood pressure were measured, hypertension developed 4 weeks after nephrectomy as expected. The magnitude of the rise in systolic blood pressure, however, was similar in both groups (nephrectomy alone, $175\pm 7.9\pm 0.3$ vs $7.4\pm 0.3$ mmol/l; creatinine $85\pm 4$ vs $80\pm 2$ $\mu mol/l$; all NS). Both parameters, however, were...
8 mmHg: nephrectomy and DPC, 171 ± 3 mmHg, NS). Since DPC had no effect on the development of hypertension in this early period, it can be concluded that the drug did not exert a direct antihypertensive effect in this model.

**Discussion**

To our knowledge, this is the first study to examine the effect of DPC on the development of experimental renal disease. The degree of renal mass reduction used was modest (two-thirds nephrectomy) to allow the animals to be studied over the 12-week period without mortality from uremia. Despite this modest reduction in renal mass, the cardinal features of the remnant kidney model, namely hypertension, proteinuria, impaired renal function and glomerulosclerosis [10] developed over the course of the study, and treatment with DPC ameliorated the evolution of renal injury. These effects are not attributable to any effect of DPC on systemic blood pressure, since this shows a similar post-operative rise in both treated and untreated nephrectomized animals. Whether such observations can be extended to other models of renal disease, which are also characterised by excess deposition of extracellular matrix, remains to be determined.

After 12 weeks, kidneys from nephrectomized rats showed only mild histological abnormalities, and it could be argued that the difference in GFR between groups 1 and 2 was far greater than would be anticipated given the small differences (Gp 1: 2.75 ± 1.01% vs Gp 2: 0.38 ± 0.26%) in the proportion of glomeruli considered to be histologically abnormal. Two explanations can be offered for this apparent discrepancy between structural and functional abnormalities. First, that only severe glomerular abnormalities (fibrinoid necrosis or severe sclerosis) were used as indices of glomerular abnormality, whilst milder abnormalities such as tuft collapse, which are not easily identified on H&E sections but may still be associated with significant functional effects, were not recorded. This would result in underestimation of glomerular pathology in the nephrectomized groups. The second explanation relates to the well-established observation that tubulo-interstitial changes are a better predictor of the degree of renal impairment than glomerular changes [reviewed in 11]. In view of the proven importance of the tubulo-interstitium in renal function, the marked differences in interstitial fibrosis, lymphocytic infiltration and tubular abnormalities between groups 1 and 2 (Table 1) are consistent with a similarly marked difference in glomerular filtration rate between the two groups.

Kidney weights were significantly lower in group 2 than group 1. The larger kidneys in group 1 may have resulted from either greater tubulo-interstitial hypertrophy (since glomerular size remained unchanged) or more interstitial water. Because of this difference, the reduction in kidney collagen per unit kidney weight in group 2 did not achieve statistical significance. Since kidneys in both groups would initially have the same collagen content, however, and final total kidney collagen was less in group 2 than group 1, it is clear that treatment with DPC prevented the deposition of collagen within the kidney.

**Table 1. Comparison of histological abnormalities in remnant kidneys from DPC-treated and untreated animals**

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytic infiltration</td>
<td>1.75 ± 0.16</td>
<td>0.5 ± 0.27</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Interstitial fibrosis</td>
<td>0.75 ± 0.16</td>
<td>0.13 ± 0.13</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Tubular atrophy</td>
<td>1.25 ± 0.31</td>
<td>0.38 ± 0.26</td>
<td>NS</td>
</tr>
<tr>
<td>Tubular casts</td>
<td>1.38 ± 0.26</td>
<td>0.88 ± 0.30</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values represent mean ± standard error of abnormality scores for each variable.

Tropocollagen is the basic structural unit of the collagen fibre, and comprises lysine- and proline-rich polypeptide chains arranged in a triple helix [12]. After secretion of tropocollagen molecules from fibroblasts, the amino groups of the lysine residues are oxidized to aldehyde groups by the enzyme lysyl oxidase, and the proline residues undergo hydroxylation to hydroxyproline, an amino acid virtually unique to collagen. The mature collagen fibre comprises multiple tropocollagen molecules, usually aligned in a ‘quarter stagger’, and stabilized by intra- and intermolecular aldimine cross-links formed from the aldehyde groups on adjacent tropocollagen molecules [13]. These cross-links are initially weak and easily disrupted, but as the collagen fibre matures they stabilize, converting soluble collagen to the insoluble form with its associated high tensile strength. [14]. Penicillamine interferes with the deposition of mature collagen fibres in three ways. The best recognized mode of action depends upon its ability to cleave aldimine cross-links, probably by forming thiazolidines with aldehyde groups on the lysine residues of tropocollagen polypeptides [15]. This mechanism cleaves only immature aldimine cross-links, however, and stabilized cross-links of mature collagen fibres are not affected. In addition to this main mechanism, studies using cultured embryonic bones have suggested that penicillamine reduces *de novo* synthesis of tropocollagen molecules [16], and analysis of punch biopsy specimens of skin from penicillamine-treated patients with scleroderma have demonstrated a reduction in hydroxyproline synthesis compared with untreated controls [7]. Since treatment with DPC influenced neither glomerular size nor systemic blood pressure in this study, it seems likely that injury was reduced by direct inhibition of intrarenal collagen deposition and consequent reduction of glomerulosclerosis and interstitial fibrosis, rather than by any effect on renal or glomerular haemodynamics. Such a mechanism could also explain the findings of Steen et al. [7] who reported that treatment with DPC significantly reduced the incidence of renal disease in patients with scleroderma, a condition characterized...
by excessive collagen deposition in both skin and viscera.

In addition to an effect on collagen metabolism, DPC may influence renal injury by two other mechanisms: first, by interaction with reactive oxygen species; and second, by its effects on inflammatory cells. Nath and Salahudeen [17] reported that rats maintained on antioxidant-deficient diets developed accelerated renal growth, reduced GFR and tubulo-interstitial injury. They also demonstrated increased urinary ammonia excretion in the anti-oxidant deficient animals, and it was proposed that accumulation of reactive oxygen species in the kidney might directly stimulate ammniogenesis, thereby activating the alternate complement pathway and inducing tissue injury. Since DPC can inhibit myeloperoxidase [18], which catalyses the reaction of H$_2$O$_2$ with Cl$^-$ to produce the highly reactive hypochlorite, and when complexed with copper can also dismute superoxide to the less reactive species in the kidney might directly stimulate ammniogenesis, thereby activating the alternate complement pathway and inducing tissue injury. Since DPC can inhibit myeloperoxidase [18], which catalyses the reaction of H$_2$O$_2$ with Cl$^-$ to produce the highly reactive hypochlorite, and when complexed with copper can also dismute superoxide to the less reactive species H$_2$O$_2$ and O$_2^-$, it could be argued that neutralization of reactive oxygen species contributes to the protective effect of DPC seen in this study. Lymphocytic interstitial infiltrates occur in all forms of nephropathy except minimal change disease, and the magnitude of interstitial infiltration correlates with the degree of renal excretory impairment [19]. T-lymphocyes stimulate fibrogenesis directly, via cytokine-mediated stimulation of fibroblasts, and indirectly, via macrophage activation and subsequent fibroblast activation [20]. The redox state of cell surface sulphhydryl groups on macrophages and T-lymphocytes exerts a significant influence over their function [21], and since DPC reduces sulphhydryl groups, and DPC-protein conjugates have been demonstrated on the surface of monocytes after exposure to DPC, this provides another potential mechanism by which treatment with this drug could influence tubulo-interstitial fibrosis and progression of chronic renal injury.

The findings of this study suggest that penicillamine may be of use in preventing renal fibrosis, the characteristic histological feature of chronic renal disease. It is well-established, however, that penicillamine can itself cause nephropathy, and reported prevalence rates of proteinuria in patients taking DPC for rheumatoid arthritis range from 7–20% [22]. In a minority, DPC-induced glomerulonephritis occurs as part of a lupus-like syndrome, or a clinical syndrome resembling Goodpasture disease, but the majority of affected patients have membranous glomerulonephritis without associated systemic symptoms [22]. In this group of patients, withdrawal of penicillamine is followed by disappearance of proteinuria after 6–8 months, and there are no reports of chronic renal failure resulting from glomerulonephritis induced by DPC. A report by Kirby et al. [23] in which DPC-associated proteinuria was found to resolve at a similar rate whether or not the drug was withdrawn, has led some authors to recommend continuation of DPC treatment in patients developing proteinuria [24]. If the effects of DPC on progression of chronic renal disease seen in the present study were confirmed in patients with chronic renal injury, further experimental studies would be needed to weigh the potential for slowing disease progression by the use of DPC against the risk of inducing glomerulonephritis in a proportion of those treated.

In conclusion, this study demonstrates that DPC significantly reduces glomerulosclerosis and interstitial fibrosis and slows the fall in glomerular filtration rate in an animal model of chronic renal injury. The precise mechanism of this effect remains to be established, but direct inhibition of mature collagen deposition seems most likely. Confirmation of a similar effect in humans would raise the possibility of a therapeutic role for DPC in the treatment of patients with chronic renal disease.

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