A steady-state labelling approach to the measurement of proteoglycan turnover *in vivo* and its application to glomerular proteoglycans

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Rats were implanted with mini osmotic pumps delivering sodium [^{35}S]sulphate and their newly synthesized proteoglycans were labelled over a 146 h period (steady-state labelling). Proteoglycan turnover was measured *in vivo* using a chase protocol. Glomerular proteoglycans were recovered quantitatively and the perlecan present was isolated by immunoprecipitation. The procedure allows newly synthesized proteoglycans to be quantified in mass units (pmol of glycosaminoglycan sulphate) after labelling and during the chase. Ultrastructural-immunogold experiments identified the location of perlecan as the glomerular basement membrane and mesangial matrix. Perlecan in the basement membrane was quantified using the ultrastructural-immunogold technique. Perlecan comprises about 10 % of the total glomerular proteoglycans, which are otherwise associated with glomerular cells and the mesangium. Both the total glomerular heparan sulphate proteoglycans and perlecan turn over rapidly ($t_{\frac{1}{2}} \sim$ 3–4 h and < 3 h respectively). In contrast, turnover of proteoglycans in other tissues was slow, except in the liver where the heparan sulphate and chondroitin sulphate $t_{\frac{1}{2}}$ values were 16 h and 9 h respectively. Microalbuminuria was induced with a low-dose regimen of puromycin aminonucleoside. At the onset of microalbuminuria (5 days) there was no change in the level of newly synthesized perlecan, or in perlecan in the glomerular basement membrane detected by immunogold labelling. Newly synthesized perlecan had undergone a minimal change in turnover rate by day 5 in puromycin aminonucleoside-treated rats. In contrast, the total glomerular proteoglycan population showed a dramatic decrease in turnover by day 5. Since there was no evidence of accumulation of glomerular proteoglycans on either day 5 or day 6, it is likely that decreased turnover of cell-associated proteoglycans is accompanied by an equivalent decrease in their synthesis.

INTRODUCTION

The extracellular matrix (ECM) of tissues is composed of a variety of proteins and proteoglycans, and plays an important role in normal physiological functions. The amount of any specific component present in an ECM represents a balance between the levels of its synthesis and turnover by the cells of the tissue. Although it is acknowledged that this balance is frequently disturbed in a number of disease processes, few attempts have been made to measure the synthesis and turnover of ECM components *in vivo*.

Previous studies by us and others have concentrated on the synthesis and turnover of renal glomerular proteoglycans, since these molecules have been implicated as key components of the glomerular basement membrane (GBM). The GBM restricts the passage of plasma proteins across it on the basis of both their size and charge [1–4]. The charge selectivity properties have been attributed to the presence of anionic proteoglycans [5,6]. The presence of heparan sulphate proteoglycan (HSPG) in the basement membrane was established by Kanwar and Farquhar [7,8], and subsequently it was shown that enzymic removal of the glycosaminoglycan chain led to increased permeability of the membrane to proteins [9]. However, the precise role of proteoglycans in selective filtration across the normal membrane and in proteinuria is still not well understood. Indeed, the actual concentration of anion charge in the normal membrane, which has been estimated at between 8 and 12 mequiv·litre^{-1} [10–12], is lower than theoretical values of the concentration (165 mequiv·litre^{-1} [13]) required for an effective anionic barrier. However, the validity of this calculation has been questioned recently [14]. The HSPGs are also likely to have important structural roles in the membrane. Their interactions with other proteins such as type IV collagen and laminin [15] may contribute to maintaining the macromolecular architecture of the membrane and therefore to its selectivity properties in filtration. Some growth factors may bind to the heparan sulphate.

Our previous studies, using *in vivo* pulse–chase procedures, suggested that the GBM HSPG is normally turned over rapidly, with a half-life of about 5 h [16]. However, pulse-labelling with [^{35}S]sulphate does not necessarily provide a quantitative basis for comparing proteoglycan synthesis and turnover in the ECM of normal and diseased animals. Plasma ^{35}S radioactivity, and therefore specific radioactivity, changes continuously. Rates of uptake into plasma and clearance from it may vary in different groups of animals, e.g. normal and nephrotic rats. Therefore we have developed a procedure to allow steady-state labelling of proteoglycans *in vivo*, quantification of the amount of newly synthesized molecules, and measurement of their turnover. We have used this to investigate the turnover of proteoglycans in the ECM of several rodent tissues. We have also investigated glomerular proteoglycan synthesis and turnover at the onset of microalbuminuria after treating rats with puromycin aminonucleoside.

MATERIALS AND METHODS

Source and treatment of animals

Female Wistar rats (160 g) were obtained from the specific pathogen-free colony in the Department of Comparative Biology, Charing Cross and Westminster Medical School, and were maintained on standard rat chow and water, *ad libitum*. In some

Abbreviations used: ECM, extracellular matrix; GBM, glomerular basement membrane; HSPG, heparan sulphate proteoglycan.

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experiments rats were injected subcutaneously with puromycin aminonucleoside (1.5 mg/100 g body weight in 0.5 ml of saline) at 24 h intervals, on 3 successive days. Control rats received saline vehicle only. In experiments to assess urinary protein loss, rats were acclimatized to metabolism cages for 4 days prior to receiving the first injection of puromycin aminonucleoside or saline, and were kept in the cages for a further 6 days to facilitate the collection of 24 h urine specimens. To assess the effect of puromycin aminonucleoside on the level of newly synthesized perlecan and other glomerular proteoglycans, rats were implanted with mini osmotic pumps containing [^{35}S]sulphate (see below) and received the first injection of the agent at the same time (day 1). Further injections were given on days 2 and 3. Rats were then killed 6 h after the third injection (day 3), and 24, 48 and 72 h later (days 4, 5 and 6 respectively). Control groups received saline vehicle only on days 1, 2 and 3, and were killed on day 3 and day 5. The weights of whole animals, their kidneys and kidney cortices, the number of glomeruli and the amount of glomerular DNA did not differ between the treated and control animals. The blood ^{35}S specific radioactivity was very similar in the treated and control animals throughout the experiment.

Steady-state labelling of glomerular and other tissue proteoglycans

Alzet mini osmotic pumps (model 2001) from Scientific Marketing were filled with 200 μl of sodium [^{35}S]sulphate (12–18 mCi/ml; Dupont Ltd.) according to the manufacturer's instructions and were incubated in 0.9 % saline overnight at room temperature prior to subcutaneous implantation into the neck scruff of rats. The saline incubation initiates osmotic pumping prior to implantation. The pumps deliver 1 μl/h (manufacturer's information). Following implantation, blood ^{35}S radioactivity was measured at intervals. Tail vein blood (20 μl) was mixed with NCS tissue solubilizer (1 ml; Amersham International), treated with hydrogen peroxide (0.2 ml; 50 %, w/v) and added to Ecoscint A scintillation solution (8 ml; National Diagnostics), prior to counting of radioactivity in a Packard liquid scintillation counter.

Animals that had been radioactively labelled for 146 h were subject to a chase procedure. The mini osmotic pump containing [^{35}S]sulphate was removed and replaced with one containing 100 mM non-radioactive sodium sulphate. At the same time a bolus of non-radioactive sodium sulphate (7.0 mM; 1 ml/100 g body weight) was injected intraperitoneally. Chase animals were killed at various intervals up to 55 h.

In vivo renal perfusion with cetylpyridinium chloride and isolation of glomeruli

The quantitative recovery of ^{35}S-labelled proteoglycans from glomeruli depends on prior perfusion of the kidneys at physiological blood pressure with cetylpyridinium chloride (0.01 %, w/v) [17]. The cetylpyridinium chloride forms an insoluble complex with proteoglycans and glycosaminoglycans, fixing them in the tissue, prior to removing the kidney and isolating the glomeruli by sieving. In the present study rats were anaesthetized (Hypnorm, 0.3 ml/kg body weight, intramuscular; diazepam, 2.5 mg/kg body weight, intraperitoneal) and their kidneys perfused, as described previously [17]. After perfusion, kidneys were removed and placed in PBS containing 0.01 % cetylpyridinium chloride and a cocktail of proteinase inhibitors [17]. The cortices were dissected out, cut into small pieces and gently passed through a series of graded stainless steel sieves (106, 180 and 53 μm pore size; Endecotte Ltd.), washing through with the same solution. Finally the glomeruli, retained on the 53 μm sieve, were washed extensively with PBS containing proteinase inhibitors, counted and divided into a number of portions for extraction.

Extraction of ^{35}S-labelled glycosaminoglycans

Glomeruli were washed with 0.05 M sodium acetate, pH 6.4, to remove proteinase inhibitors and digested overnight with papain at 65 °C [18]. Other tissues from steady-state-labelled animals were chopped and digested with papain. Digests were cooled to room temperature and treated with NaOH (0.5 M final concentration) for 1 h to ensure complete solubilization of glycosaminoglycans. Neutralized digests were divided into three portions. One was analysed directly for [^{35}S]glycosaminoglycan radioactivity by Sephadex G-25 chromatography (PD10 columns; Pharmacia) [19]. A second was treated with freshly prepared cold nitrous acid, pH 1.5, to degrade heparan sulphates [20]. The ^{35}S-labelled chondroitin sulphates remaining after this procedure were separated by Sephadex G-25 chromatography and counted for radioactivity. A third portion was digested with chondroitinase ABC to degrade chondroitin sulphate [21] and the digest was analysed for ^{35}S-labelled heparan sulphates by Sephadex G-25 chromatography.

Extraction of ^{35}S-labelled macromolecules and immunoprecipitation of perlecan

Glomeruli were extracted twice with 4 M guanidine hydrochloride, 0.05 M sodium acetate, 4 % (w/v) CHAPS, containing proteinase inhibitors, pH 5.8, at 4 °C. The residue was extracted twice with 8.0 M urea, 0.05 M Tris/HCl, pH 8.6, at 4 °C. The procedure, which effectively solubilizes all glomerular proteoglycans, was carried out as described previously [16]. Extracts were analysed for ^{35}S-labelled macromolecules by Sephadex G-25 chromatography [19].

A monospecific polyclonal antiserum (R48) was used to immunoprecipitate the combined glomerular extracts. The antiserum reacts specifically with a basement membrane HSPG [22] identified subsequently as perlecan [23]. The immunoprecipitation method [16] was adapted from that of Ledbetter et al. [24]. Immunoprecipitated [^{35}S]proteoglycans were solubilized in 0.01 M phosphate buffer, 2 % SDS and 0.1 M dithiothreitol, pH 7.4, at 100 °C for 3 min, and radioactivity was counted in a β-scintillation counter.

Two other antisera, AH-GBM and NTS, raised in rabbits against whole human and whole rat GBM respectively (M. Davies, unpublished work), were also used to immunoprecipitate glomerular extracts. Antisera AH-GBM and NTS were preadsorbed against human and rat red cells respectively. In order to compare the [^{35}S]proteoglycans precipitated by R48, AH-GBM and NTS, glomerular extracts were prepared from rats that had been pulse-labelled for 7 h with sodium [^{35}S]sulphate [17]. The immunoprecipitated [^{35}S]proteoglycans were separated by chromatography on a dissociative Sepharose CL-4B column [25].

Immunogold electron microscopy and image analysis

Following renal perfusion with cetylpyridinium chloride, approximately six 2 mm^2 pieces of each kidney cortex were cut out and fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, at 4 °C for 3 h. After fixation the specimens were embedded in Lowicryl K4M and ultrathin sections were prepared for electron microscopy [26]. Sections on nickel grids were immersed in primary antiserum (R48, AH-GBM or NTS) diluted 1:10 with PBS containing 0.1 % (w/v) sodium azide, 0.1 % (w/v) BSA and 0.05 % (v/v) Tween 20 for 2 h and then, after washing,

in secondary antibody [goat anti-rabbit IgG conjugated to 10 nm gold particles (Janssen Pharmaceuticals Ltd., Wantage, U.K.), diluted 1:10, as above]. After further washing, grids were stained with 1% aqueous uranyl acetate and then Reynolds lead citrate [26]. Controls consisted of sections processed in the absence of primary antibody or in the presence of non-immune serum.

Sections were examined with a Philips EM 300 transmission electron microscope at 60 kV and micrographs taken of all the GBM areas which satisfied three criteria: (a) the area was readily identified as GBM and distinguishable from the mesangial matrix, (b) the area was free of and at least 1 μm away from any holes in the section, (c) only GBM with non-obliquely sectioned epithelial cell foot processes and endothelium was used. A micrograph of a diffraction grating replica (Agar Scientific Ltd.) was taken in conjunction with each section for calibrating magnification in the subsequent image analysis.

Photographic prints of micrographs were placed on an XY co-ordinate digitizer tablet (Calcomp) and the perimeters of each portion of GBM were traced with an electronic pen connected to a dedicated computer system and software (Computer Automation Inc.) to calculate the area of GBM. The pen was then used to count the number of gold dots in each segment of GBM. The data were placed in a spreadsheet (Microsoft EXCEL) and the mean immunogold dot density per unit area of GBM was calculated. An analysis of variance programme (one-way SPSSX) was used to evaluate the data.

Analytical methods

Urinary albumin was assayed using a single radial immunodiffusion method [27]. Antibody to rat albumin was obtained from Nordic Ltd. The Lowry–Folin method was used to measure high concentrations of protein (> 80 mg/l) in the urine [28]. The serum inorganic sulphate concentration was determined by precipitation with ^{133}BaCl [29]. Prior to measurement of serum [^{35}S]sulphate in a Packard β-scintillation counter, serum (100 μl) was mixed with trichloroacetic acid (20%, w/v; 50 μl) to precipitate serum proteins. Glomerular DNA was measured after digestion with proteinase K, using a modified diaminobenzoic acid (DABA) assay [30].

RESULTS

In vivo steady-state labelling of proteoglycans with [^{35}S]sulphate

Blood ^{35}S radioactivity increased rapidly after implanting the mini osmotic pumps, reaching a peak level within 12 h. Thereafter it decreased to about 80% of the maximum value by 24 h, and subsequently it remained relatively constant up to 146 h after implanting the pump (Figure 1). The period from 24 to 146 h was designated the steady-state labelling period. The overshoot in the first 12 h of labelling may be due to differences in osmotic pressure between the saline bath used for preincubation of the pump and the rats' subcutaneous tissue, or to acute local inflammatory changes at the site of implantation.

The chase procedure was effective in rapidly clearing [^{35}S]sulphate from the blood following the labelling period. The serum inorganic sulphate concentration remained constant throughout the chase period (0.43 ± 0.05 mM, mean $\pm$ S.E.M.). Both serum total ^{35}S radioactivity and the specific radioactivity of [^{35}S]sulphate were reduced to about 20% of initial values by 17 h and remained at this level throughout the 55 h chase period.

To establish an appropriate level of [^{35}S]sulphate for labelling of glomerular proteoglycans during the steady-state procedure, rats were implanted with mini pumps delivering different amounts of ^{35}S radioactivity. Incorporation of ^{35}S into glomerular proteoglycans was measured after 146 h of labelling, and increased linearly with delivery of between 2.5 and 20 μCi of [^{35}S]sulphate/h. Thereafter levels of ^{35}S incorporation decreased, so that with 28 μCi/ h the radioactivity in ^{35}S-labelled macromolecules was only 75% of that achieved with 20 μCi/h (results not shown). This effect is likely to be due to radiotoxicity [31]. Thus all subsequent experiments were carried out with pumps filled with 12–18 mCi/ml sodium [^{35}S]sulphate, below the level of radioactivity which might lead to toxic effects.

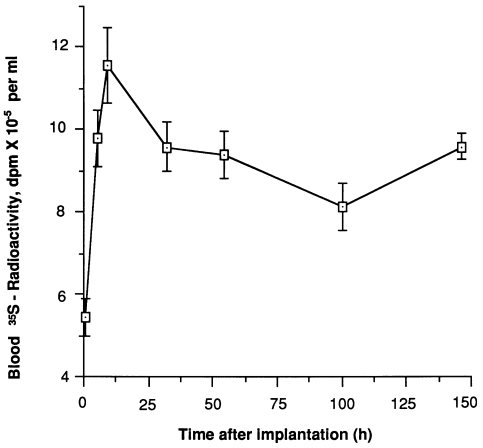

Figure 1 Blood [^{35}S]sulphate levels

Rats were implanted with mini osmotic pumps filled with sodium [^{35}S]sulphate (12 mCi/ml), and blood radioactivity was measured at intervals. Results are means $\pm$ S.E.M. ($n = 6$, except at 104 and 146 h where $n = 3$).

Table 1 Quantification of newly synthesized glomerular glycosaminoglycans at the end of the steady-state labelling period

Rats were implanted with mini osmotic pumps containing 18 mCi/ml [^{35}S]sulphate and were labelled over a period of 146 h. Glomeruli were isolated and the amount of inorganic sulphate incorporated into glycosaminoglycans calculated as described in the text. Measurements are the means $\pm$ S.E.M. for 24 rats. The renal cortex from both kidneys was pooled for each rat.

Renal cortex per rat, wet wt. (g)	1.11 ± 0.02
Total glomeruli per rat (n)	19093 ± 513
Serum sulphate concentration (mM)	0.43 ± 0.05
$10^{-9} \times$ Serum ^{35}S radioactivity (d.p.m./l)	3.27 ± 0.31
$10^{-3} \times$ Serum ^{35}S specific radioactivity (d.p.m./nmol of sulphate)	7.50 ± 1.01
$10^{-3} \times$ Total glomerular [^{35}S]glycosaminoglycans (d.p.m./10^4 glomeruli)	7.05 ± 0.67
Total glomerular glycosaminoglycan sulphate (nmol)	1.79

The level of newly synthesized proteoglycans was measured in glomeruli following a 146 h steady-state labelling period (Table 1). The cellular precursor for the addition of sulphate to glycosaminoglycans during their biosynthesis is 3′-phosphoadenosine 5′-phosphosulphate, and the ^{35}S specific radioactivity of this metabolite will be in equilibrium with, and equal to, that of serum [^{35}S]sulphate during prolonged steady-state labelling. Thus the amount of newly synthesized proteoglycan in glomeruli can be quantified in terms of mol of sulphate incorporated into glycosaminoglycans by measuring the [^{35}S]glycosaminoglycan radioactivity and the serum ^{35}S specific radioactivity (Table 1). Of the glomerular glycosaminoglycans, 70% are HSPGs and 30% are chondroitin or dermatan sulphate

Table 2 Quantification of newly synthesized proteoglycans in non-renal tissues at the end of the steady-state labelling period

Rats were implanted with mini osmotic pumps containing 12 mCi/ml [^{35}S]sulphate and were labelled over a period of 146 h. Tissues were analysed for [^{35}S]glycosaminoglycans and the amount of inorganic sulphate incorporated into them was calculated as described in the text. Results are means ± S.E.M., $n = 4$ rats.

	Glycosaminoglycan synthesis (nmol of SO_4/mg wet tissue wt.)		
Tissue	Total	Heparan sulphate	Chondroitin sulphate
Costal cartilage, anterior half	1470 ± 110	60 ± 10	1180 ± 90
Costal cartilage, posterior half	1820 ± 150	210 ± 20	1400 ± 110
Liver	40.2 ± 3.3	19.8 ± 1.5	17.8 ± 1.4
Lung	53.0 ± 3.2	12.3 ± 2.1	19.9 ± 4.5
Muscle	4.6 ± 0.8	0.8 ± 0.1	2.7 ± 0.5
Skin	92.3 ± 5.2	12.5 ± 0.6	66 ± 4.1

proteoglycans (results not shown), and this agrees well with results obtained by radioactivity measurements after *in vivo* ^{35}S-pulse-labelling experiments [17].

The renal cortex forms about 85% of the mass of the whole rat kidney. In separate *in vivo* labelling experiments, glomerular [^{35}S]glycosaminoglycans formed 5% of the total [^{35}S]glycosaminoglycan in the whole cortex. Thus, based on the data in Table 1, newly synthesized proteoglycan in the renal cortex would account for about 42 nmol of sulphate/mg wet tissue weight. This is similar to the amount of newly synthesized glycosaminoglycan present in the tissues of two other complex organs, liver and lung (Table 2). However, other tissues, e.g. cartilage and muscle, contain widely differing amounts of newly synthesized glycosaminoglycan. With the exception of the liver and lung, which contain prominent amounts of heparan sulphate, the predominant glycosaminoglycans in non-renal tissues are chondroitin and/or dermatan sulphates.

Turnover of glomerular and other tissue ^{35}S-labelled proteoglycans

Glycosaminoglycans isolated from proteoglycans and measured at the end of the steady-state labelling period are those synthesized, but not yet catabolized, during this period. Thus, following steady-state labelling, chase experiments were carried out to assess the relative rate of turnover of glycosaminoglycans in different tissues (Figure 2). Glomerular proteoglycans were turned over markedly more rapidly than those in other tissues. About 70% of the total glomerular [^{35}S]glycosaminoglycans

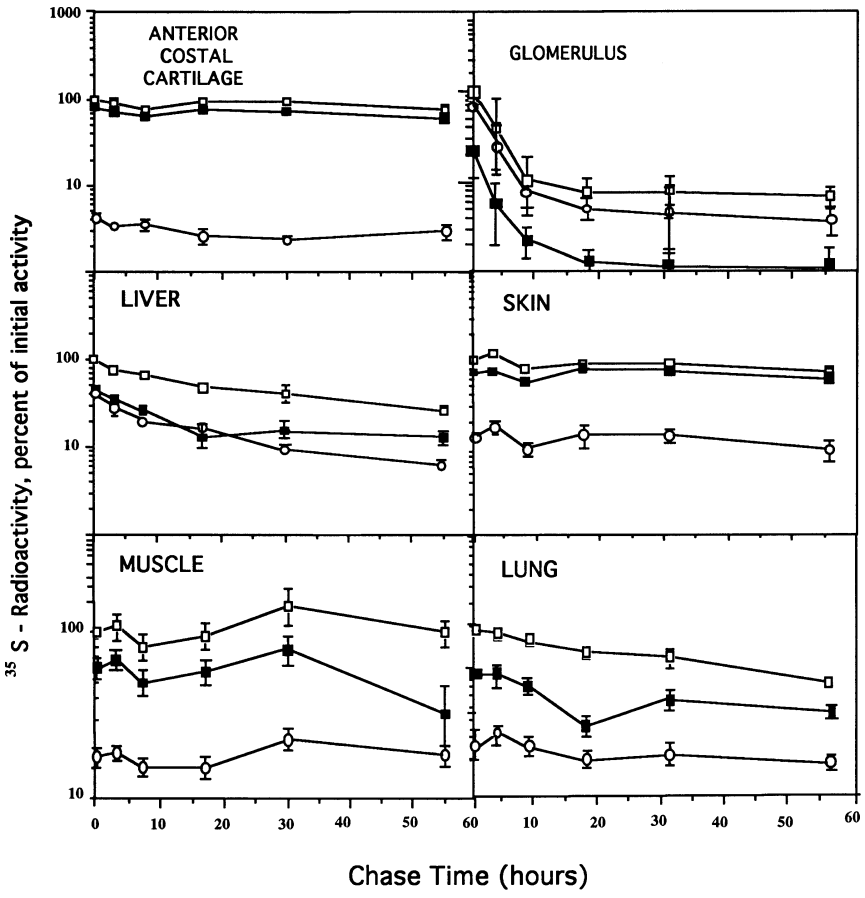

Figure 2 Turnover of ^{35}S-labelled glycosaminoglycans in various tissues

Newly synthesized glycosaminoglycans were labelled *in vivo* using the steady-state protocol over 146 h. This was followed by an *in vivo* chase procedure. [^{35}S]Glycosaminoglycans remaining in the tissues at various intervals of the chase were estimated. □, Total glycosaminoglycans; ○, heparan sulphate; ●, chondroitin sulphate. Results are means ± S.E.M., $n = 4$ animals per chase time. Note the log scale of the ordinate.

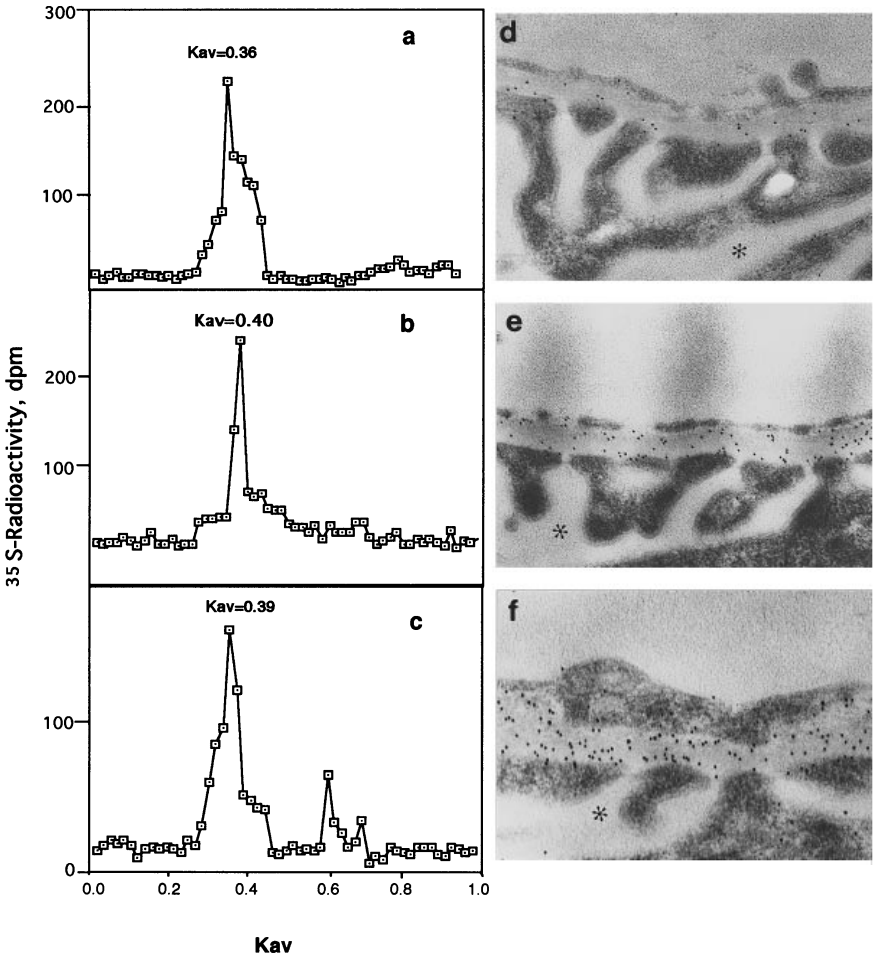

Figure 3 GBM proteoglycans precipitated from glomerular extracts

Rats were pulse-labelled with [^{35}S]sulphate, and glomeruli were isolated and extracted with guanidine hydrochloride and urea. Extracts were precipitated with three different antisera, (**a**) R48, (**b**) AH-GBM and (**c**) NTS, and the resolubilized precipitates were chromatographed on a dissociative Sepharose CL-4B column (**a**–**c**). Ultrastructural-immunogold labelling shows the presence of epitopes reacting with the R48 (**d**), AH-GBM (**e**) and NTS (**f**) antisera across the GBM. The asterisk (*) indicates the urinary space. Magnifications are $\times$29250 (**d**), and $\times$38350 (**e**, **f**).

undergo turnover during the first 8 h of the chase. Both heparan sulphates and chondroitin sulphates are catabolized. Heparan sulphate remaining after this time turns over at such a slow rate that it could not be accurately measured. Very little chondroitin sulphate remains after $\sim$ 10 h.

In contrast, the turnover of glycosaminoglycans in most other tissues was very slow. Little or no turnover was detected in cartilage, lung, muscle or skin during the 55 h chase. Glycosaminoglycans in the liver had a biphasic pattern of turnover with a fairly rapid initial phase (heparan sulphate, $t_{\frac{1}{2}} = 16$ h; chondroitin sulphate, $t_{\frac{1}{2}} = 9$ h).

Identification of GBM HSPGs

Several different proteoglycans are present in the rat glomerulus [17]. The antiserum R48 [22] was used to precipitate GBM HSPGs. To investigate the nature of the proteoglycans precipitated by R48, rats were pulse-labelled with [^{35}S]sulphate, their kidneys perfused with cetylpyridinium chloride, and the glomeruli isolated and extracted. Glomerular extracts were immunoprecipitated with R48, and the precipitates solubilized and chromatographed on a dissociative Sepharose CL-4B column (Figure 3). Glomerular extracts were also immunoprecipitated with two other antisera, AH-GBM and NTS. The AH-GBM and NTS antisera have specificity for a broad spectrum of GBM components, whereas R48 was raised against a specific basement membrane proteoglycan [22] and reacts with perlecan [23]. Nevertheless, all the antisera immunoprecipitated a ^{35}S-labelled proteoglycan of the same size (Figures 3a–3c). Each antiserum

Table 3 Onset of microalbuminuria after injection of puromycin aminonucleoside

Rats were acclimatized to metabolic cages for 3 days and then injected subcutaneously with puromycin aminonucleoside on days 1, 2 and 3. All urine was collected for each 24 h interval following the first injection and analysed for albumin. Results are means $\pm$ S.E.M., $n = 3$ rats for each time point.

Time after first injection (days)	Urinary albumin (μg/24 h)
Pre-injection	0.07 $\pm$ 0.03
2–3	0.10 $\pm$ 0.06
3–4	0.25 $\pm$ 0.14
4–5	0.70 $\pm$ 0.33
5–6	2.36 $\pm$ 0.90

Table 4 Gold particle densities for perlecan over the GBM after injection of puromycin aminonucleoside to induce microalbuminuria

Puromycin aminonucleoside (PA) or saline vehicle (C) was injected on days 1–3. Treated and control animals were killed 6 h after the final injection (day 3), and on days 4–6. Perlecan was detected using antiserum R48. The density of gold particles is expressed as the number of particles/μm^2 area of GBM. Two rats were analysed per time point and results are shown as the means ± S.E.M. for the total number of sections analysed. Analysis of variance showed no differences between groups at the $P < 0.05$ level.

Time after first injection (days)	Treatment	Gold particles (no./μm^2 of GBM)	Number of areas of GBM analysed
3	C	58 ± 2	99
3	PA	51 ± 2	60
4	PA	56 ± 11	28
5	PA	64 ± 12	28
6	C	54 ± 10	28
6	PA	65 ± 4	24

was used to immunoprecipitate glomerular extracts from four different rats. Each precipitated a similar proportion of the total glomerular ^{35}S-labelled macromolecules (R48, 9.1 ± 1.4 %; AH-GBM, 9.2 ± 1.8 %; NTS, 11.3 ± 2.0 %; means ± S.E.M., $n = 4$). Further addition of antisera did not precipitate any more [^{35}S]proteoglycan. In ultrastructural-immunogold experiments each antiserum bound to epitopes that were localized in the GBM and mesangium (Figures 3d–3f). There was no evidence for intracellular or cell membrane localization of epitopes. Control sections incubated with non-immune serum showed no gold particles over any GBM, although occasional non-specific immunogold staining was seen in the capillary lumens (results not shown). The results suggest that R48 precipitates, quantitatively, the major HSPG of the GBM and mesangium, and that this proteoglycan is a form of perlecan. They do not exclude the possibility that other proteoglycans may also reside in the GBM.

Quantification of perlecan in the GBM in normal and puromycin aminonucleoside-treated rats

The R48 antiserum was used with the steady-state labelling technique to quantify newly synthesized perlecan in normal rats and during the onset of microalbuminuria following puromycin aminonucleoside treatment. Significant microalbuminuria occurred on days 4–5 after the first injection of puromycin aminonucleoside and increased thereafter (Table 3). There was no difference in the level of newly synthesized perlecan between normal rats (1.3 ± 0.3 pmol of glycosaminoglycan sulphate/μg of glomerular DNA; $n = 4$) and in rats with microalbuminuria (1.3 ± 0.41 pmol/μg; $n = 4$) on day 5 of the experiment. Furthermore, no significant differences were found in treated animals prior to the onset of microalbuminuria (days 3 and 4), or immediately after it (day 6).

We also measured perlecan in the GBM during the onset of microalbuminuria using the R48 antiserum and quantitative ultrastructural-immunogold investigations. The number of gold particles per μm^2 area of GBM was counted over the period of onset of microalbuminuria and in saline-injected controls (Table 4). Analysis of variance showed no significant differences between

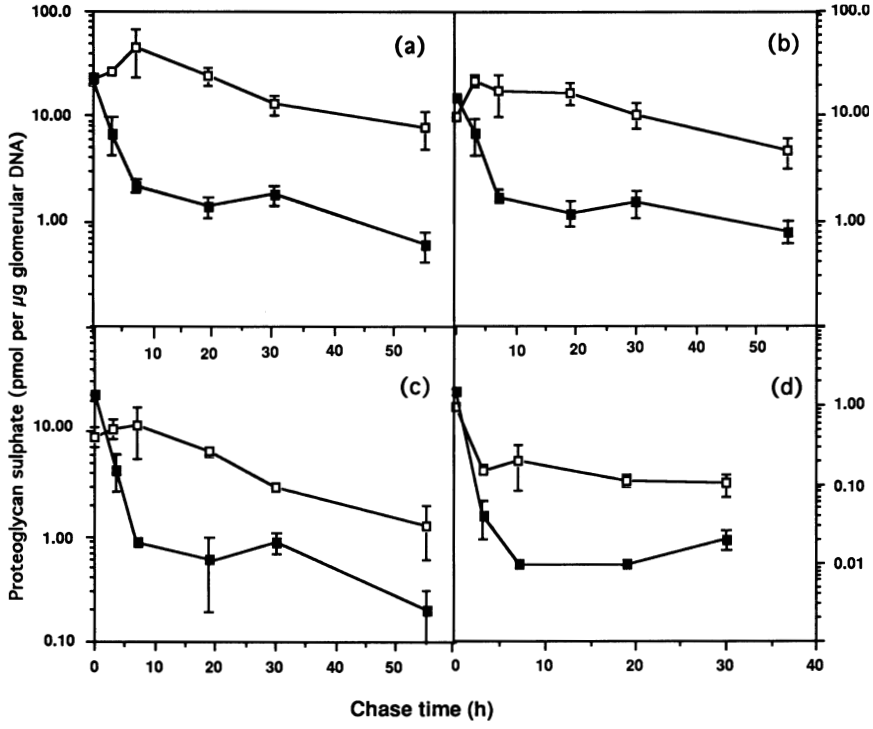

Figure 4 Metabolic turnover of glomerular proteoglycans in normal rats and in rats treated with puromycin aminonucleoside

Rats were labelled using the steady-state protocol from day 1, on which they also received the first injection of either puromycin aminonucleoside (□) or saline (■). After further injections on days 2 and 3, a chase experiment was carried out on day 5. Results are means ± S.E.M. ($n = 4$ rats for each chase time). (**a**) Total glomerular sulphated macromolecules; (**b**) total glomerular sulphated glycosaminoglycans; (**c**) total glomerular heparan sulphate; (**d**) perlecan. Note the log scale of the ordinate.

any two groups of puromycin aminonucleoside-treated rats or between them and the controls.

Turnover of perlecan in normal and puromycin aminonucleotide-treated rats

The turnover of perlecan was investigated in normal rats and in puromycin aminonucleoside-treated animals on day 5 after the first injection, when significant microalbuminuria occurred. Rats were subjected to steady-state labelling as above, and on day 5 the mini-pumps were removed and the chase procedure initiated and continued for 55 h, as detailed in the Materials and methods section. The turnover of the total glomerular sulphated macromolecules, glycosaminoglycans, heparan sulphate and perlecan was measured. The turnover of the total sulphated macromolecules and the glycosaminoglycan population in the normal glomerulus were both biphasic (Figures 4a and 4b). About 80–90 % of these molecules were rapidly catabolized, their turnover being completed by about 7 h into the chase, whereas the remaining 10–20 % turned over much more slowly. It was not possible within the time frame of the chase to derive an accurate $t_{\frac{1}{2}}$ for this population, but it is likely to be in excess of 50 h. The total heparan sulphate pool, which makes up about 70 % of the total glycosaminoglycan pool, followed a similar turnover pattern, with a $t_{\frac{1}{2}}$ of $\sim$ 3–4 h (Figure 4c). The perlecan pool, isolated by immunoprecipitation with R48, also turned over rapidly, with a $t_{\frac{1}{2}}$ of $<$ 3 h (Figure 4d). Over 95 % of the pool had turned over completely by 7 h, with the remainder undergoing little or no further turnover within the time frame of the experiment.

The quantitative amounts of all proteoglycan fractions in the glomerulus of puromycin aminonucleoside-treated rats did not differ significantly from those found in the control rats on day 5, as is evident from the zero chase time data (Figure 4). However, the turnover of proteoglycans in the treated rats was significantly different from that in the normal rats. For the total sulphated macromolecule fraction, the glycosaminoglycan fraction and the heparan sulphate fraction, there was a complete loss of the initial rapid turnover phase, while the slow phase of metabolism was maintained. As a result, the heparan sulphate pool, for example, turned over with a $t_{\frac{1}{2}}$ of $\sim$ 24 h (Figure 4c). The turnover of perlecan in the treated rats was also retarded, but to a lesser extent. About 90 % of this proteoglycan was metabolized during the first 3 h of the chase in treated rats, whereas over 95 % was removed in normal animals (Figure 4d). Thereafter there was no further rapid turnover in treated rats. Since the rapid phase continued in normal rats, only about 1 % of the newly synthesized perlecan remained after 7 h of chase, but 10 % remained in treated animals.

DISCUSSION

Previously we used an *in vivo* pulse–chase protocol to measure the turnover of GBM heparan sulphate in adult rats [16]. This indicated a rapid turnover rate ($t_{\frac{1}{2}} \sim 5$ h). However, the design of pulse–chase experiments has several drawbacks. Firstly, they are liable to label predominantly the most rapidly turning over molecules of any given population. Secondly, such experiments do not indicate whether the molecules so measured constitute a minor or major proportion of the whole population. Thirdly, the plasma and cellular levels of the radiolabelled precursor molecule are not in a steady state and the results of such experiments are, therefore, semi-quantitative at best. These limitations make rigorous comparisons between groups difficult, e.g. between control and nephrotic animals. The rate of absorption, plasma specific radioactivity and rate of excretion of the precursor may differ between different groups [32].

In order to avoid these problems, we have developed a steady-state labelling method, measuring newly synthesized proteoglycan in terms of a mass unit rather than a radioactivity unit. A constant plasma ^{35}S specific radioactivity is maintained for a period of days. The specific radioactivity of cellular ^{35}S-labelled 3′-phosphoadenosine 5′-phosphosulphate pools will equilibrate with it, since cells use extracellular inorganic sulphate if the supply is not restricted [33]. Even when cells are maintained in culture in media with a very low inorganic sulphate content, most have only a limited capacity to use alternative sulphate sources such as cysteine [33]. Thus the steady-state labelling protocol provides a quantitative measurement of newly synthesized proteoglycan and, when coupled with the chase procedure, relates this to proteoglycan turnover *in vivo*.

The long labelling period of the steady-state method would allow all proteoglycans in the basement membrane to be labelled with [^{35}S]sulphate. Two antisera (AH-GBM and NTS) of broad specificity for GBM components and the R48 antiserum all precipitated similar proportions of ^{35}S-labelled HSPG from total glomerular extracts. Moreover, the [^{35}S]proteoglycans precipitated by these antisera all had the same hydrodynamic size. This, together with the immunolocalization experiments, indicates that the perlecan proteoglycan that is precipitated by R48 [23] is a major HSPG of the rat GBM and mesangial matrix. However, it is of smaller hydrodynamic size (K_{av} 0.36–0.40) than the perlecan found in many basement membranes and in the Engelbreth–Holm–Swarm mouse sarcoma [34,35]. Typically these larger forms have a K_{av} of $\sim$ 0.20 on Sepharose CL-4B chromatography. Klein et al. [36] precipitated a rat GBM HSPG with an antiserum against Engelbreth–Holm–Swarm sarcoma large HSPG, a proteoglycan subsequently recognized as perlecan. The immunoprecipitated GBM HSPG had a smaller core protein (molecular mass 150–200 kDa) than that of perlecan found in other tissues (400–450 kDa). Further experiments using pulse labelling led to the proposal that rat glomerular cells synthesize a large perlecan core protein which is rapidly processed by proteolysis to give smaller core proteins [36]. This concept is consistent with our *in vivo* findings.

The steady-state labelling/chase experiments show that about 95 % of perlecan has a very rapid turnover in normal rats ($t_{\frac{1}{2}} <$ 3 h). This turnover rate is similar to that found previously using the pulse–chase protocol ($t_{\frac{1}{2}} \sim 5$ h). The close proximity of these measurements, obtained with two different experimental designs, adds further weight to the proposal that perlecan in the glomerulus turns over very rapidly. The R48 antiserum and the immunogold procedure detected antigen associated only with the GBM and mesangial matrix, and not with epithelial, endothelial or mesangial cells. Thus the small half-life of the proteoglycans precipitated with this antibody represents turnover of extracellular molecules and is not due to an intracellular pool of newly synthesized molecules that are metabolized without even leaving the cell.

The present data show that glomerular proteoglycans generally, and not just those of the GBM, have a rapid turnover rate. This is much faster than that of proteoglycans in several other tissues investigated and may relate to the function of glomerular proteoglycans. In the case of GBM proteoglycans involved in forming an anionic barrier to the diffusion of cationic plasma proteins, the barrier may have to be renewed continually to prevent clogging. This would require a rapid turnover of the GBM and mesangial proteoglycans, especially if they are normally present in low concentrations. Our data show that the amount of proteoglycan present in the GBM represents only 10–12 % of the total glomerular proteoglycan. Plasma membrane proteoglycans probably account for the majority of the glom-

erular proteoglycans, and it is likely that most of those labelled by the steady-state method are cell-associated. It is not known to what extent these proteoglycans contribute to the anionic barrier, if at all. However, *in vitro* studies indicate that cell membrane proteoglycans generally exhibit rapid turnover rates [37], and cell culture experiments on glomerular epithelial cells support this [38].

The steady-state label/chase protocol allowed us to directly compare the level of newly synthesized perlecan and its turnover in the glomerulus in the puromycin aminonucleoside model of nephrotic syndrome with that in normal rats. In particular we investigated the glomerular proteoglycan status at the onset of microalbuminuria in this model. A low daily dose of puromycin aminonucleoside was used to avoid early excessive toxicity towards the glomerular epithelial cells. High doses lead to the detachment of podocyte foot processes from the GBM [39]. Electron microscopy did not show any foot process detachment during the 6-day duration of our experiments (results not shown), but significant microalbuminuria occurred on day 5. At this time our biochemical analysis showed no difference in the amount of newly synthesized perlecan in the glomerulus compared with controls, and there was only a minimal decrease in turnover of these proteoglycans (Figure 4d). Moreover, the density of perlecan in the GBM detected by immunogold analysis was unchanged on day 5 in puromycin aminonucleoside-treated animals compared with controls (Table 4). Thus it seems unlikely that the onset of microalbuminuria is related to the loss of perlecan in the GBM at this stage of the disorder. There is some controversy regarding whether loss of anionic sites occurs in the GBM during the later stages of puromycin aminonucleoside-induced nephrosis [40–44].

In contrast to perlecan, the total newly synthesized glomerular proteoglycans showed a marked decrease in turnover rate on day 5 of the experiment in treated animals compared with that in controls. A prolonged decrease in catabolism would be expected to lead to increases in the total glomerular proteoglycans on day 5 unless it is accompanied by an equivalent decrease in synthesis of these proteoglycans. The pre-chase data in Figure 4(c) suggest that this might occur, but this was not proven statistically. As noted above, a large proportion of the total glomerular proteoglycans are likely to be cell-surface-associated. Whether they contribute to the glomerular permeability barrier to albumin is unknown.

In conclusion, the steady-state label/chase protocol has allowed us to make quantitative measurements of the total glomerular proteoglycans and of perlecan, and to determine their turnover rates in normal rats and at the onset of microalbuminuria in experimental nephrosis. The method has refined our earlier finding that there is a very rapid turnover of glomerular proteoglycans generally, and of perlecan in particular. Moreover, this method can distinguish subtle changes in proteoglycan metabolism in experimental renal disease.

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