Pentosan Polysulfate Decreases Proliferation and Net Extracellular Matrix Production in Mouse Mesangial Cells

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Abstract. Glomerulosclerosis is characterized by extracellular matrix accumulation and is often associated with mesangial cell proliferation. Heparin-like molecules have been shown to decrease glomerulosclerosis in vivo, although their cellular site and mechanism of action is still unclear. In this study, a line of glomerular mesangial cells derived from normal mice was used to determine whether pentosan polysulfate (PPS) inhibited proliferation and altered extracellular matrix turnover. Cells treated with PPS showed a decrease in cell number beginning 24 h after treatment, which was maintained for 5 d. For matrix accumulation and degradation studies, cells were treated for 5 d and collagen types I and IV protein were measured by enzyme-linked immunosorbent assay as well as matrix metalloproteinases (MMP) measured by zymography. Collagen types I and type IV were significantly decreased in the media (P < 0.0001) and cell layer (P < 0.005) after treatment with PPS but not after treatment with heparin. By zymography, MMP-2 was significantly increased after treatment with PPS (P < 0.001) and heparin (P < 0.05). PPS and heparin also decreased MMP-9 (P < 0.001) after treatment. Reverse zymography showed the presence of tissue inhibitors of metalloproteinases (TIMP)-1 and -2 in control mesangial cells. Treatment with PPS and heparin increased TIMP-1. In addition, TIMP-3 was found in the medium of treated but not control cells. In conclusion, PPS alters extracellular matrix turnover through the induction of MMP-2 and alterations in the TIMP profile and may be useful in decreasing progressive glomerulosclerosis.

Human and experimental glomerulosclerosis is characterized by a gradual increase in glomerular mesangial extracellular matrix (ECM) (1–4). The turnover of mesangial matrix is normally slow, and several investigators have postulated that an imbalance between accumulation and degradation of ECM could contribute to the development of glomerulosclerosis.

Heparin and heparin-like molecules have been shown to decrease the proliferation of several types of cells in vitro, including mesangial cells (5), smooth muscle cells (6,7), and glomerular epithelial cells (8). In addition, heparin and/or low molecular weight heparin fragments have been shown to slow or prevent the progression of glomerulosclerosis in vivo (5,9–12). The mechanism of action on net ECM production in vivo is unclear. However, heparin decreases collagen synthesis by smooth muscle cells in vitro (13). Finally, it has recently been shown that heparin influences the induction of metalloproteinase (MMP) production in vitro, with an inhibitory effect in some cell types (14) or a stimulatory effect in others (15).

Heparin is not commonly used in the treatment of glomerulosclerosis because of its anticoagulant properties, and low molecular weight heparin preparations, which have reduced anticoagulant activity, must be parenterally administered. Recently, an oral pentosan polysulfate (PPS) preparation, essentially devoid of anticoagulant activity, has become available (Elmiron®). This product has been shown to decrease glomerulosclerosis in a mouse model of glomerulosclerosis (12). To determine the cellular site of action, we chose to study glomerular mesangial cells because they are a major contributor to ECM turnover (16–18).

Materials and Methods

Cell Culture and Experimental Conditions

Glomerular mesangial cells were isolated from normal 4-wk-old mice (C57B1/6J X SJL/J) and maintained as described previously (19). Except for growth curves, the experiments were performed in six-well plates (Nunc Nalge International, Naperville, IL). The cells were plated so that the cell number in each well was approximately the same at the end of the experiment and that the cells were semi-confluent (approximately 150,000 cells/well). Briefly, the cells were plated in medium with 20% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD) and allowed to attach. The serum concentration was decreased to 0.1% overnight before each experiment, and the following day the cells were exposed to fresh medium containing 0.1% FBS alone or with the addition of 100 μg/ml PPS (Elmiron®, IVAX Corp., Miami, FL) or heparin (sodium salt, Sigma, St. Louis, MO). PPS or heparin was added each day for 5 d. At the end of the
5 d, media was collected for the assessment of metalloproteinases or collagen production, and the cell layer was used to determine cell number, prepare RNA, or measure collagen accumulation.

For growth curves, cells were plated at equal density in medium containing 20% serum in 24-well plates. Twenty-four hours later, 0.1% FBS-containing medium with or without increasing concentrations of PPS (5 to 100 μg/ml) were added to assess dose–response. Subsequent growth curves were performed with PPS (100 μg/ml) or heparin (100 μg/ml) or 0.1% FBS-containing medium. Cell number was assessed on days 1, 3, and 5.

**Media and Cell Layer Collection**

**Metalloproteinase Production and RNA Preparation.** On the day of collection, medium from those wells designated for zymography was centrifuged to remove cell debris and frozen at −70°C for later use. The cell number from these same wells was determined by direct cell counting, and the remaining cells were used to prepare total RNA by using Tri Reagent® (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s directions. The protocol was modified by the addition of muscle glycogen (5 PRIME-3 PRIME, Inc., Boulder, CO) as a nonspecific carrier to increase the yield of total RNA. The final RNA pellet was dissolved in 10 μl of diethyl pyrocarbonate water and stored at −70°C.

**Collagen Analysis.** The media and cell layers of separate but parallel wells were collected for determination of α1 (I) and α1 (IV) collagen at the same time as zymography and RNA analysis. Ascorbic acid (50 μg/ml) and β-aminopropionitrile (80 μg/ml) were added daily to the cells in culture. As previously described (18), the medium was collected and centrifuged to remove cell debris, and the supernatant was stored with protease inhibitors (ethylene diaminetetracetic acid, phenylmethylsulfonyl fluoride, N-ethylmaleimide) at −70°C. The cell layer was collected in 1 ml of 5 M guanidine, 0.1 M Tris-HCl, pH 8.6, and protease inhibitors. Cell numbers were determined by direct cell counts in parallel wells.

**Collagen Assay by Enzyme-Linked Immunosorbent Assay.** Medium was incubated for 24 h in a 96-well Nunc-Immuno Maxisorp plate (Nalge Nunc International, Naperville, IL) followed by washing and blocking with 3% bovine serum albumin/phosphate-buffered saline for an additional 24 h. A total of 100 μl of an antibody against collagen type IV (1:750) or collagen type I (1:1000) (Biodesign, Kennebunk, ME) was applied for 1.5 or 2 h, respectively. After five washes with 0.05% Tween/phosphate-buffered saline, biotinylated goat anti-rabbit IgG (Sigma) was applied for 1.5 h. After five washes, streptavidin alkaline phosphatase followed by p-nitrophenyl phosphate was used for color development. The plate was read in a Titertek Multiskan plate reader at 405 nm. Conditions were identical to those described previously (18), except that the standard curves were modified. The concentrations of the type I (Collaborative Biomedical Products, Bedford, MA) standards were 5 to 40 ng/well and the type IV standards 4 to 32 ng/well. The curves were linear (r = 0.98).

**Collagen Production.** Mesangial cells were plated as described above in 6-well plates and maintained with or without PPS for 5 d. On the fourth day of treatment, the medium was removed and proline-free Dulbecco’s modified Eagle’s medium with 0.1% FBS was added to each well and incubated for 4 h. After this washout period, the medium was changed to proline-free Dulbecco’s modified Eagle’s medium containing 0.1% FBS, 10 ng/ml ascorbic acid, and 5 μCi/well of [1, 2, 3-H]proline (Amersham, Arlington Heights, IL), and PPS in those cells being treated. The medium and cell layer were collected 24 h later and processed according to Diegelmann and Peterkofsky (20), using purified bacterial collagenase (Sigma). Collagen production was determined on quadruplicate wells processed individually from two separate experiments. Cell number was determined on duplicate wells from treated and control cells.

**Zymography for Metalloproteinases**

The amount of MMP-9 and MMP-2 gelatinases in the medium was assessed by using 10% zymogram gels (Novex, San Diego, CA). Briefly, medium was diluted to normalize for cell counts (approximately 50,000 cells/ml) before 5% Laemmli buffer (without β-mercaptoethanol) was added to each sample. After electrophoresis, the gels were washed for 1 h in 2.5% sodium dodecyl sulfate and incubated overnight in 50 mM Tris buffer as described previously (18). The gels were stained with Coomassie blue and air-dried. Gels were also incubated in 50 mM Tris buffer, with the addition of 25 mM ethylenediaminetetraacetic acid to check for nonmetalloproteinase-dependent bands of proteolytic activity. Densitometry was performed using NIH image 1.6 to quantify the relative gelatinase activities.

**Reverse Zymography**

TIMP were assessed by reverse zymography in the supernatant and the cell layer, as described (21). Briefly, gels containing gelatinase A were prepared. Medium was diluted to normalize for cell number as described for zymography. Gels were washed for 1 h in 2.5% sodium dodecyl sulfate after electrophoresis and incubated overnight at 37°C. Coomassie blue staining and air-drying were described for zymography. To quantify the TIMP activity, densitometry was performed using NIH Image 1.6.

**Reverse Transcription and mRNA Determination**

One microgram of total RNA was reversed-transcribed, as described previously, using a First Strand cDNA synthesis kit (Boehringer Mannheim, Indianapolis, IN) (18). The reversed-transcribed material was diluted to a working concentration of 33 ng/μl. A typical PCR reaction contained 66 ng of reversed-transcribed material in a final volume of 50 μl. Standard PCR was performed for MMP-9, MMP-2, and α1 (I) collagen using primers previously published (18, 22, 23). Competitive PCR was performed for α1 (IV) collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described previously (23). The gels were photographed using an IS-1000 Digital Imaging System and saved as a Tiff file. Densitometry was performed using the computer program NIH image 1.6. The standard curves for competitive PCR were generated as described (22, 23). For standard PCR, the densitometry units were divided by the number of attomoles of GAPDH to normalize for the differences between samples.

**Statistical Analyses**

Data from three independent experiments were analyzed. Differences between PPS treatment, heparin, and control cells were measured using the t test or one-way ANOVA (Prism, GraphPad, San Diego, CA).

**Results**

**Dose Response and Growth Curve**

Cells were treated for 4 d with increasing concentrations of PPS. Proliferation decreased in a dose-dependent manner (Figure 1A). Cell number was equally suppressed after the addition of PPS (100 μg/ml) or heparin (100 μg/ml) (Figure 1B). Cell number decreased 24 h after the addition of PPS or heparin. By day 3, cell number was 50% of control and this difference was maintained up to 5 d in the presence of either agent. Viability...
was not affected, as assessed by trypan blue staining. The antiproliferative effect of 5 d of PPS treatment was reversible by reexposing the cells to medium containing 20% FBS (data not shown).

Collagen Determination

The amount of collagen types I and IV was determined in media and cell layers by enzyme-linked immunosorbent assay. There was a significant decrease of $\alpha_1$ (I) collagen in the medium ($P < 0.0001$) and monolayer ($P < 0.005$) of cells treated with PPS (Figure 2). $\alpha_1$ (IV) collagen was also significantly decreased in the medium and cell layer ($P < 0.0001$) in cells treated with PPS (Figure 3). Heparin did not have a significant effect on collagen type I or IV in the media or cell layer.

Effects of PPS on Collagen Synthesis and Production

The percentage of collagen-sensitive material in semiconfluent cells (150,000/well) was equal in cells treated with PPS (28.6 ± 4.3%; $n = 3$) and control (26.1 ± 2.42%; $n = 4$). Experiments performed on sparse cells revealed similar results (cells treated with PPS, 21.98 ± 2.32, $n = 4$; control cells, 21.53 ± 2.59, $n = 4$).

Zymography

There was an increase in secreted, soluble MMP-2 (72-kD gelatinase) (Figure 4) after treatment with PPS (E, $P < 0.001$) and heparin (H, $P < 0.05$) compared with control (C). There was also an increase in the active form of this gelatinase most notably with PPS (Figure 4A, lower band). In contrast, MMP-9, the 92-kD gelatinase (Figure 4C) was markedly decreased in the presence of PPS and heparin ($P < 0.001$).
Reverse Zymography

Analysis of TIMP by reverse zymography showed the presence of TIMP-1 and -2 in the medium of mouse mesangial cells (Figure 5). Treatment of cells with PPS significantly upregulated TIMP-1 \( (P < 0.0001) \). Heparin also increased TIMP-1 \( (P < 0.01) \). Treatment had no statistically significant effect on TIMP-2 release. TIMP-3, however, was only detectable in the medium of cells treated with PPS or heparin.

mRNA Determination

\( \alpha 1 \) (I) collagen was not significantly affected by treatment with PPS. By competitive PCR, the ratio \( \alpha 1 \) (IV)-collagen:GAPDH mRNA did not differ between treated cells and control cells (Figure 6, \( P = NS \)). Figure 7 shows a graph of PCR results for MMP-9. Cells treated with PPS had a significantly lower \( (P < 0.05) \) MMP-9 mRNA level than did control cells. Heparin also significantly lowered mRNA levels of MMP-9 \( (P = 0.001, \text{data not shown}) \). There was no significant difference in MMP-2 mRNA levels between PPS and control mesangial cells.

Discussion

The administration of the heparinoid PPS to a line of mouse glomerular mesangial cells significantly modified ECM synthesis and degradation. It also decreased proliferation. We found a significant decrease in type I and IV collagen proteins in the medium and the cell layer after 5 d of treatment with PPS. Heparin has also been shown to decrease synthesis of type I and type IV collagen in chondrocytes and vascular smooth muscle cells \( (13) \, \text{in vitro} \, (24) \). Surprisingly, the heparin preparation we used did not significantly decrease colla-

Figure 3. \( \alpha 1 \) (IV) collagen synthesis. PPS treatment \( (\square) \) induced a significant reduction of \( \alpha 1 \) (IV) collagen synthesis in the medium and cell layer \( (P < 0.0001) \) as measured by enzyme-linked immunosorbent assay, compared with control cells \( (\square) \). Data are expressed as percentage of control of triplicate experiments. Medium (top: PPS, \( n = 12 \); heparin, \( n = 7 \); control, \( n = 11 \)) and cell layer (bottom: PPS, \( n = 8 \); heparin, \( n = 4 \); control, \( n = 7 \)).

Figure 4. Matrix metalloproteinase-2 (MMP-2; 72-kD gelatinase) and MMP-9 (92-kD gelatinase). (A) Representative zymogram of mesangial cells treated with PPS (E), heparin (H), or control cells (C). S, standard. (B) Analysis of triplicate experiments showing an increase of MMP-2 (PPS, \( \square, n = 10, P < 0.001 \) versus control; heparin, \( \square, n = 5, P < 0.05 \) versus control; control, \( \square, n = 7 \)). (C) There is a decrease of MMP-9 in triplicate experiments (PPS, \( \square, n = 8 \); heparin, \( \square, n = 5, P < 0.001 \) versus control; control, \( \square, n = 6 \)) in mesangial cells.
gen synthesis in our experiments, although it was as effective as PPS in inhibiting cell proliferation. Thus, as we have shown before, there is not a tight correlation between cell proliferation and ECM synthesis (23). It has been shown that antiproliferative activities vary widely between commercial sources of heparin (25), and the heparin preparation we used has been reported to have an intermediate capability to inhibit cell growth (26).

Although PPS significantly lowered type I and type IV collagen net protein levels in both the cell monolayer and conditioned media, there was no significant change in their mRNA levels. This finding suggests that either the secretion of collagen or its extracellular stability was affected. Heparin has not been shown to decrease collagen type I and IV mRNA and protein levels in human mesangial cells (4), although it did decrease their levels in smooth muscle cells (13). Thus, the effects of heparin may differ depending on the vascular bed from which the cells are isolated. Therefore, it may be necessary to compare human smooth muscle cells isolated from various vascular beds of the same individual. In addition, it may be useful to compare different individuals, since we have shown the mesangial sclerosis is largely dependent on genetic background in mice.

Because the data suggested that PPS may alter ECM turnover, we investigated the effect of PPS and heparin on MMP and TIMP activity as detected by zymography and reverse

Figure 5. Reverse zymography. (A) Representative reverse zymogram. (B) Densitometric analysis of duplicate experiments for tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2. Cells treated with the following: PPS (E), n = 7; heparin (H), n = 6; control cells (C), n = 6. S, standard. *P < 0.05; **P < 0.01.

Figure 6. Competitive PCR of α1 (IV) collagen mRNA levels. Cells treated with PPS (■, n = 5) versus control cells (□, n = 5) from triplicate experiments were reverse-transcribed, as described in Materials and Methods. Competitive PCR was performed for α1 (IV) collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results were expressed as attomoles of α1 (IV) collagen divided by GAPDH. Data are represented as the ratio of α1 (IV) collagen and GAPDH (P = not significant).

Figure 7. PCR data comparing PPS-treated (■) and untreated (□) cells. MMP-9 mRNA levels were significantly lower (P < 0.05) in cells treated with drug for 5 d. The data are expressed as percentage of control for each experiment. Data were analyzed from triplicate experiments (PPS, n = 7; control, n = 8).
zymography. We and others have shown that human (27), mouse (18,28), and rat mesangial cells (29) produce MMP-2 and MMP-9 in vitro. Both of these MMP degrade nonhelical type IV collagen and denatured interstitial collagen (30,31). By zymography, PPS significantly decreased MMP-9, and increased both the pro and active forms of MMP-2. The current hypothesis regarding the activation of MMP-2 (30) is that proMMP-2 is activated on the cell surface by the membrane type matrix metalloproteinase (MT-MMP-1) in conjunction with TIMP-2 (30,31). Because the mesangial cells treated with PPS had a decrease in net release of types I and IV collagens without change in the corresponding mRNA levels, the effect was likely attributable to an increase in active MMP-2 in the extracellular space. Recently, Butler et al. (32) have shown that the complex of MT-MMP-1/TIMP-2 is required for activation of proMMP-2. An excess of TIMP-2, however, could inhibit this activation. By reverse zymography, we did not detect an increase in TIMP-2. Our conclusion of increased local activation of MMP-2 is consistent with these data and is further supported by the observation that there were no differences in the amount of collagenase-sensitive material between treated and control cells. These data suggest that the effect of PPS on ECM turnover occurs, at least in part, at the extracellular level.

The synthesis of MMP-9 and MMP-2 have been shown to be independently regulated (30–33). This was most likely the case in the current experiments. The decrease in MMP-9 protein and mRNA levels after PPS and heparin treatment suggests that this effect was exerted at the transcriptional level. Our finding of a significant increase of TIMP-1 after treatment with PPS or heparin may be an additional independent action of these drugs or may result from the decrease in MMP-9 protein. In contrast, it appears that regulation of MMP-2 protein occurs mainly at the level of proenzyme activation rather than the transcriptional level. It is possible that PPS could directly influence proMMP-2 binding to the cell surface if one compares its effect with those of heparin. Heparin has been shown to influence the C-terminal interactions between TIMP-2 and proMMP-2, which may in turn influence cell surface binding (32,34).

Finally, PPS caused a shift of TIMP-3 into the medium from the ECM. TIMP-3 is normally bound to the ECM, whereas TIMP-1 and TIMP-2 are soluble. The present data suggest that one of the effects of PPS may be to facilitate the degradation of collagens by MMP by removing TIMP-3 from the cell surface.

This differential effect of PPS and heparin on MMP-2 and MMP-9 production is consistent with previous data describing the effects of heparin on MMP as cell-type specific (14,15). Kenagy et al. reported that heparin inhibited the production of MMP-9 without affecting MMP-2 in primate arterial smooth muscle cells (14).

We found that increased levels of MMP-2 in normal mouse mesangial cells were associated with decreased cell proliferation. We have previously shown in mesangial cells isolated from mice transgenic for bovine growth hormone that increased MMP-2 levels were also associated with decreased cell proliferation (18). This effect may be species-specific, because others have found that decreased cell proliferation was associated with decreased MMP-2 levels in rat mesangial cells (35,36) as well as in rabbit aortic smooth muscle cells (37). Turck et al. (35) used antisense techniques to block MMP-2 in cloned lines of rat mesangial cells, which produced only MMP-2, and found that they became quiescent after MMP-2 inhibition. Other investigators using synthetic inhibitors of MMP-2 were also able to inhibit proliferation in rabbit aortic smooth muscle cells and rat mesangial cells (36,37).

MMP play an important role in vivo in facilitating turnover of ECM components. Because PPS seems to affect ECM by selective activation of MMP-2, this may be one mechanism by which it could act therapeutically in slowing progressive glomerulosclerosis.

References

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