Effect of heparin on mesangial cell growth and gene expression of matrix proteins

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Abstract

Background. Mesangial cell (MC) proliferation and matrix expansion are characteristic features of many glomerulopathies. Heparin has been shown to inhibit MC proliferation in vitro and mitigate cell proliferation, matrix expansion, proteinuria, renal insufficiency, and hypertension in experimental glomerulonephritis and subtotal renal ablation. We examined the effect of standard heparin on MC proliferation and matrix protein expression in vitro which necessarily excludes the confounding influences of haemodynamic, inflammatory, haemostatic, and various other processes that are present in vivo.

Methods. Gene expression and release of fibronectin (FN), collagen IV and laminin by cultured rat MC were tested in the presence and absence of heparin. In addition, the effect of transforming growth factor-β1 (TGF-β1) on the gene expression of those matrix proteins was assessed.

Results. Within a 3–1000 μg/ml concentration range, heparin inhibited gene expression and release of FN by 10% fetal calf serum (FCS)-stimulated MC in a concentration-dependent manner. At concentrations of 300 and 1000 μg/ml, heparin inhibited fibronectin mRNA levels in TGF-β1 (6 ng/ml) stimulated cells. However, heparin had no effect on gene expression or release of collagen IV or laminin under these conditions. Heparin markedly inhibited 10% FCS-stimulated MC proliferation in a concentration-dependent manner.

Conclusions. Heparin inhibited MC growth and fibronectin production. These effects may, in part, account for the reported beneficial effects of heparin on the course of renal disease in experimental animals.

Key words: collagen, laminin, fibronectin, TGF-β1

Introduction

Mesangial cell (MC) proliferation and/or mesangial matrix expansion are characteristic features of many different glomerulopathies. Several earlier studies have revealed that standard heparin, endogenous heparin-like substances and heparan sulfate can inhibit MC proliferation in vitro [1–4]. Likewise, heparin administration has been reported to lessen MC proliferation and matrix protein accumulation in rats with anti-thy 1.1-induced mesangioproliferative glomerulonephritis independently from its anticoagulant action [5]. Recently, heparin has been shown to inhibit IL-1β-mediated upregulation of gene expression of the broad-substrate spectrum metalloproteinase, transin in cultured MC [6]. Moreover, heparin has been shown to suppress collagen IV gene expression in MC cultured in media containing high glucose concentrations [7]. In addition, heparin administration has been found to attenuate proteinuria and hypertension and to retard progression of renal insufficiency and glomerulosclerosis in the remnant kidney of rats with subtotal renal ablation and streptozotocin-induced diabetes mellitus [8–10]. In the present study, we examined the gene expression and release of matrix proteins in cultured rat MC in order to isolate the direct effect of heparin from those mediated by a myriad of haemodynamic, inflammatory, and haemostatic events contributing to glomerulosclerosis and hypertension in vivo.

Subjects and methods

Materials

Heparin, RPMI 1640 medium, trypsin, and fetal calf serum (FCS) were purchased from Sigma Chemical Co. (St Louis, MO). Transforming growth factor-β1 (TGF-β1) was purchased from R and D Systems Inc. (Minneapolis, MN). The following products were purchased from Chemicon International Inc. (Temecula, CA): rat fibronectin (FN) and laminin, human collagen IV, rabbit anti-rat antibodies against FN and laminin, and rabbit anti-human collagen IV antibody. Peroxidase labelled goat anti-rabbit IgG antibody and O-phenylenediamine (OPD) substrate kits were purchased from Zymed Laboratories Inc. (San Francisco, CA). Tissue culture plates and flasks were purchased from Corning Corporation (Pleasanton, CA). ELISA plates were purchased from Fisher Scientific Co. (Tustin, CA).
Mesangial cell preparations

Rat glomeruli were isolated from renal cortices by differential sieving using the technique described by Harper et al. [11]. In brief, the kidneys were excised from Sprague–Dawley rats weighing 100–150 g and placed in ice-cold phosphate-buffered saline (PBS). Renal cortices were minced and passed through 40- and 150-mesh sieves successively. Glomeruli were then collected on a 200-mesh sieve, washed three to four times with ice-cold PBS, resuspended and placed in 75 ml tissue culture flasks containing RPMI 1640 medium supplemented with 20% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml Na-selenite. D-valine was substituted for L-valine in the medium to exclude fibroblasts from the cell population, and the cell suspension was incubated at 37 °C in 5% CO₂ and 95% air. Once the primary culture reached confluence (25–30 days), subcultures were carried out every 7–10 days. On each occasion, confluent cells were detached by trypsinization (1% trypsin, 0.4% EDTA) prior to reculturing. The culture media were renewed at intervals of 48 h throughout the incubation period. Cells between 6 and 20 passages were used in these experiments.

The identity of MC cells and purity of cell population was confirmed based on the following characteristics: (i) typical large stellate and spindle-shaped cells with numerous irregular cytoplasmic projections which formed hillocks on long-term cultures observed by phase-contrast light microscopy; (ii) cell resistance to toxic effects of puromycin (10 μg/ml); and (iii) positive immunocytochemical staining for characteristic cytoskeletal filaments such as actin, myosin and desmin and negative staining for factor VIII.

Cell proliferation studies

Cell proliferation was studied by measurement of DNA synthesis using [³H]thymidine incorporation and confirmed by cell count. To this end, MC were cultured in 96- and 24-well plates at densities of 5000 and 15 000 cells/well, respectively, in the presence of 10% FCS. After 24 h, the media were renewed with those containing either 0.5 or 10% FCS, and various concentrations of heparin (0.0–1000 μg/ml). The plates were then incubated for 24–48 h, after which the media of the 96-well plates were aspirated and cells washed twice with PBS then trypsinized as described above. The cells were subsequently harvested onto glass-fibre filter mats using an automatic cell harvester (Brandel, Rockville, MD). The filters were then placed in 7 ml scintillation vials with 5 ml of bio-safe NA solution and radioactivity measured in a liquid scintillation counter (Model LS9000, Beckman Instruments Inc., Fullerton, CA). Counting efficiency was estimated by an internal standard.

In parallel experiments, the above procedures were repeated in the absence of [³H]thymidine for quantitation of cell counts. The cells were washed twice with PBS, trypsinized and counted using a cytometer.

Matrix protein assays

Fibronectin (FN), collagen IV and laminin levels were determined in the supernatant using an inhibition ELISA as reported previously [12]. Briefly, for FN assay, flat-bottom microtitre plates were coated with 200 μl of 1000 ng/ml FN in carbonate buffer, pH 9.5. Wells were washed and incubated with 100 μl of a 1:1000 dilution of a monospecific rabbit anti-FN antibody (Abi), which had been preincubated at 37 °C for 90 min with the supernatant. After incubation for 60 min at room temperature, wells were washed three times with PBS–0.05% Tween buffer and incubated for 30 min at room temperature with 100 μl of a 1:2000 dilution of a peroxidase-labeled goat anti-rabbit IgG antibody. After washing, wells were incubated with OPD, and the intensity of color change was determined by absorption at 490 nm in an ELISA spectrophotometer (Flow Laboratories Inc., Mclean, VA). The results of the assay were compared with the mean of duplicate wells from an eight point standard curve constructed as described above, but substituting known concentrations of purified rat FN for the unknown samples.

cDNA probes

The probe used for fibronectin 1 mRNA is a 1000 bp cDNA to human fibronectin mRNA. A 1800 bp cDNA to the z1 chain of human collagen IV mRNA was used for measurement of collagen IV mRNA. A 2400 bp cDNA to synaptic laminin mRNA was used for determination of laminin mRNA. A 1100 bp cDNA to human β-actin was used to normalize for any differences in RNA loading. All probes were purchased from American Type Culture Collection (ATCC) and were labeled with [³²P]dCTP (3 000 Ci/mmol, NEN Research Products, Boston, MA) by the random primer method (Promega Prime-A-Gene System).

Northern blot analysis

MC from passages 5–10 were grown in the presence of 10% FCS until subconfluence was attained. The cells were then brought to quiescence by lowering the FCS content of the medium to 0.5%. Twenty four hours later, the media were renewed with those containing either 0.5 or 10% FCS, or TGF-β1 (6 ng/ml), and various concentrations of heparin (3, 30, 300, 1000 μg/ml). The plates were then incubated for 24–48 h after which total cellular RNA was isolated from MC by a modification of the method of Chomczynski et al. [13] using RNAzol-B (Tel-Test Inc., Friendswood, TX). The amount of RNA was quantitated by absorbance at 260 nm. Equal amounts of total RNA (15 μg) from heparin-treated and control MC were fractionated by electrophoresis on a 1% agarose/2.2 M formaldehyde gel. RNA was then transferred to nylon membrane (Bio-Rad, Richmond, CA) by capillary blotting method, and fixed by baking at 80 °C for 2 h. Subsequently, the membranes were prehybridized for 2 h at 65 °C and then hybridized with the ³²P-labelled cDNA probes for fibronectin, collagen IV, and laminin mRNA for at least 16 h in a solution containing 3 × Denhard’s reagent, 5 × SSPE, 1% SDS and 100 μg/ml salmon sperm DNA (Sigma, St Louis, MO). Membranes were washed to high stringency (0.1% × SSPE/1% SDS) twice, for 15 min each, and autoradiography with intensifying screens (−70 °C) was performed. The density of each band was measured by laser densitometer (Molecular Dynamics, Sunnyvale, CA). The same membrane was stripped for 30 min in 1% SDS, and rehybridized with actin probes, which were used to normalize for any differences in RNA loading.

Statistical analyses

Data are presented as mean ± SEM. Student’s t-test, repeated measure analysis of variance (ANOVA), Duncan’s multiple
results

Matrix proteins

At concentrations between 3 and 1000 µg/ml, heparin significantly depressed fibronectin mRNA and protein production by FCS-stimulated MC. The inhibitory effects of heparin on gene expression and release of fibronectin were dose-dependent (Figure 1).

Collagen IV mRNA levels remained unchanged in the presence of escalating concentrations of heparin. Similarly, collagen IV release by MC was unaffected by heparin within the tested concentration range of 0–1000 µg/ml (Figure 2). As with collagen IV, heparin had no significant effect on either laminin mRNA or laminin release to the extracellular media (Figure 3).

MC incubated with TGF-β1 (6 ng/ml) exhibited an increase in fibronectin and collagen IV mRNA abundance. In contrast, laminin mRNA abundance was not affected by TGF-β1. Heparin significantly depressed fibronectin mRNA levels in TGF-β1-stimulated cells.

Proliferative response

Data are shown in Figure 6. As expected, MC exhibited a marked proliferation in the presence of 10% FCS. This was evidenced by a significant rise in cell count coupled with an increased thymidine incorporation into DNA. Heparin at the concentration range between 3 and 1000 µg/ml inhibited 10% FCS-induced proliferation of MC in a dose-dependent manner. At 1 µg/ml concentration, heparin had no significant effect on the proliferative response to 10% FCS by cultured rat MC. DNA synthesis in FCS-stimulated MC was negatively related to heparin concentration (r = −0.99, P < 0.001).

Discussion

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Discussion

The present study revealed that addition of heparin to the culture media leads to a marked and dose-dependent inhibition of FCS-induced proliferation of cultured rat MC. These observations are consistent with the results of the earlier studies demonstrating growth inhibitory effect of standard heparin as well as heparan sulfate and other endogenous heparin-like substances on MC in vitro [1–4,14]. Likewise, our results agree with the reported observation that heparin
Inhibition of mesangial cell matrix production

Fig. 3. Representative northern blot of mesangial cell laminin (Lam) and the corresponding actin (Act) obtained in the presence of various concentrations of heparin (lanes 1–6 of (A) reflect measurements obtained in the presence of heparin at 0, 1, 3, 30, 300 and 1000 µg/ml), the corresponding laminin/actin mRNA (B) and laminin protein release to the culture media (C). Data are given as mean±SEM for four experiments.

Fig. 4. Representative northern blot of mesangial cell fibronectin (Fn) mRNA, collagen IV (Col) mRNA, laminin (Lam) mRNA and the corresponding actin (Act) mRNA in the presence of 0.5% FCS (lane 1), 0.5% FCS plus TGF-β1 (6 ng/ml, lane 2) and heparin at 30 (lane 3), 300 (lane 4) and 1000 (lane 5) µg/ml. Heparin administration mitigates MC proliferation in rats with anti-thy 1.1-induced mesangioproliferative glomerulonephritis [5] and those with Habu snake venom-induced focal mesangioproliferative glomerulonephritis [15].

Cellular proliferation is usually accompanied by expansion of the mesangial matrix in various proliferative glomerulonephritides [16]. Moreover, accumulation of mesangial matrix leading to obliteration of glomerular capillary loops represents the principal lesion in diabetic glomerulopathy as well as glomerulosclerosis of various etiologies. TGF-β1 has been identified as a mediator of chronic renal fibrosis in a variety of experimental and human kidney disorders [17]. This effect is thought to be largely due to its regulatory function in extracellular matrix production. Induction of TGF-β1 in the diseased glomerulus results in complex tissue reactions resulting in increased extracellular matrix production, ultimately contributing to glomerulosclerosis [18]. Blocking the actions of TGF-β1 with decorin, a natural inhibitor of TGF-β1, has been shown to inhibit the development of sclerosis and reduce proteinuria in experimental glomerulonephritis [19,20]. Heparin has been used as a treatment modality in a variety of experimental glomerular disorders. In an earlier study, Floege et al. showed that heparin administration mitigates both cell proliferation and mesangial matrix expansion in anti-thy 1.1-induced glomerulonephritis [5]. In addition, heparin administration has been shown to retard progression of renal failure and glomerulosclerosis in rats with subtotal renal ablation and prevent or reverse the histologic changes and functional alterations of diabetic nephropathy in streptozosin-treated rats [7,8,10]. These observations prompted the present study to discern the effect of heparin on the gene expression and release of...
Fig. 6. 10% FCS-stimulated cell proliferation (cell count) (A), and thymidine incorporation (B), in the presence of various concentrations of standard heparin. Data are given as mean ± SEM for six experiments. *P<0.01 (multiple measure ANOVA and Duncan’s multiple range test).

the principal constituents of mesangial matrix, i.e. fibronectin, collagen and laminin after stimulation with TGF-β1 and FCS.

Our results showed that at concentrations ≥3 μg/ml, heparin significantly inhibits production of fibronectin mRNA in FCS-or TGF-β1-stimulated cultured rat MC. The heparin-induced inhibition of fibronectin mRNA production was associated with a parallel reduction in fibronectin release into the extracellular medium. This observation suggests that standard heparin’s inhibitory effect on fibronectin production occurs at the level of transcription. In contrast with FCS, TGF-β1 failed to induce an increase in laminin gene expression in MC, confirming previous reports in fibroblasts and epithelial cells [21]. However, both TGF-β1 and FCS stimulation resulted in significant increases in collagen IV mRNA. Interestingly, heparin had no discernible effect on either the transcripts or release of laminin or collagen IV by cultured rat MC under the present experimental conditions. The inhibitory action of heparin on fibronectin production by MC observed in our in vitro experiments supports the effect reported with heparin administration in rats with anti-thy 1.1 glomerulonephritis and on progression of renal disease in rats with subtotal renal ablation and diabetic nephropathy [6–8,10]. However, in contrast with the repeated heparin administration, which has been shown to mitigate accumulation of collagen and laminin in these in vivo models, no such effects could be demonstrated in our in vitro experiments. Others have reported similar effects in smooth muscle cells [22]. Interestingly, Caenazzo et al. [7] have reported that heparin did not modify the expression of type IV collagen by cultured MC, unless the cells were exposed to high glucose concentrations. The reason for the disparity between the in vitro and in vivo effects of
heparin on collagen and laminin production is uncertain. However, it may be due to the presence of highly complex pathophysiological processes which are operative in vivo and absent in vitro. These include the participation of haemodynamic disturbances (hyper-tension and hyperfiltration), inflammatory reactions (involving leukocytes, complement system and cytokines), blood coagulation system, and macromolecular trafficking, which can singularly or together stimulate production or modify the turnover of these matrix proteins in vivo. Thus, while supporting the results of in vivo studies with regards to the inhibitory effect of heparin on fibronectin, the lack of discernible effect on collagen and laminin production in vitro does not negate the in vivo effect of heparin in pathological conditions marked by mesangial matrix accumulation.

The mechanism by which heparin produces a protective effect on a variety of glomerular lesions is unknown. The anti-proliferative effects reported by a variety of investigators, and confirmed in the present study, may be partially responsible. Some have speculated that heparin’s anticoagulant effects may be important [23], while others have suggested that the restoration of glomerular basement membrane charges by these polyanionic compounds may play a significant role [24]. Finally, heparin’s complex effects on extracellular matrix synthesis and degradation may be instrumental in its effect on glomerular sclerosis [6,7,14,25,26]. The mechanisms by which heparin inhibits fibronectin gene expression are unknown, and its effect on matrix expansion is poorly understood. The effect of FCS on cell matrix expansion can be mediated by a variety of factors, including TGF-β1. Heparin, at high concentrations, was shown to inhibit the effects of TGF-β1 in vitro, suggesting that at least in part, its effects on extracellular matrix production may be mediated through inhibition TGF-β1.

In conclusion, the study revealed that heparin can significantly lower TGF-β1 and FCS-induced gene expression and release of fibronectin in cultured rat MC. The study further confirmed that heparin inhibits the proliferative response to FCS in a dose-dependent manner. These effects of heparin may, partly, account for the reported favourable actions of heparin on the course of renal disease in humans and experimental animals.

References


Received for publication: 26.2.98 Accepted in revised form: 17.4.98