Heparin Prevents Intracellular Hyaluronan Synthesis and Autophagy Responses in Hyperglycemic Dividing Mesangial Cells and Activates Synthesis of an Extensive Extracellular Monocyte-adhesive Hyaluronan Matrix after Completing Cell Division*

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Background: Hyperglycemic dividing mesangial cells initiate intracellular hyaluronan synthesis, autophagy, and cyclin D3-mediated hyaluronan matrix formation.

Results: Heparin prevents the intracellular responses but induces synthesis of a monocyte-adhesive extracellular hyaluronan matrix after cell division.

Conclusion: Heparin inhibits inflammatory responses in diabetic glomeruli.

Significance: Responses of dividing cells to hyperglycemia contribute to diabetic pathologies.

Growth-arrested rat mesangial cells (RMCs) at a G0/G1 interphase stimulated to divide in hyperglycemic medium initiate intracellular hyaluronan synthesis that induces autophagy/cyclin D3-induced formation of a monocyte-adhesive extracellular hyaluronan matrix after completing cell division. This study shows that heparin inhibits the intracellular hyaluronan synthesis and autophagy responses, but at the end of cell division it induces synthesis of a much larger extracellular monocyte-adhesive hyaluronan matrix. Heparin bound to RMC surfaces by 1 h, internalizes into the Golgi/endoplasmic reticulum region by 2 h, and was nearly gone by 4 h. Treatment by heparin for only the first 4 h was sufficient for its function. Streptozotocin diabetic rats treated daily with heparin showed similar results. Glomeruli in sections of diabetic kidneys showed extensive accumulation of autophagic RMCs, increased hyaluronan matrix, and influx of macrophages over 6 weeks. Hyaluronan staining in the glomeruli of heparin-treated diabetic rats was very high at week 1 and decreased to near control level by 6 weeks without any RMC autophagy. However, the influx of macrophages by 6 weeks was as pronounced as in diabetic glomeruli. The results are as follows: 1) heparin blocks synthesis of hyaluronan in intracellular compartments, which prevents the autophagy and cyclin D3 responses thereby allowing RMCs to complete cell division and sustain function; 2) interaction of heparin with RMCs in early G1 phase is sufficient to induce signaling pathway(s) for its functions; and 3) influxed macrophages effectively remove the hyaluronan matrix without inducing pro-fibrotic responses that lead to nephropathy and proteinuria in diabetic kidneys.

Mesangial expansion is the principal glomerular lesion in diabetic nephropathy (DN)2 that reduces the area for filtration and leads eventually to sclerosis and renal failure (1, 2). However, the mesangial extracellular matrix expansion and sclerosis are preceded by a phenotypic activation and transient proliferation of the glomerular mesangial cells, followed by a prominent glomerular infiltration of monocytes and macrophages (3, 4). Glomerular monocytes and macrophages have been prominently identified in DN in both animal models (4) and humans (5) and appear to have a key role in the induction of mesangial matrix expansion, hypercellularity, and the onset of proteinuria (6, 7). The molecular mechanisms underlying glomerular infiltration and activation by monocytes in DN are still unclear.

Previous studies (8) as well as our own (9, 10) have shown that there is a significant increase in hyaluronan matrix in glomeruli during the 1st week after induction of diabetes in rats by streptozotocin, coincident with glomerular monocyte/macrophage influx (4). There is compelling evidence for a causal link between increased glomerular hyaluronan matrix and monocyte/macrophage accumulation. In diabetic glomeruli, the structure of the hyaluronan matrix can mediate monocyte adhesion and activation, thereby contributing directly to the sclerotic process.

Hyaluronan is a linear glycosaminoglycan composed of repeating disaccharide units of N-acetylglucosamine and d-glucuronic acid with alternating β-1,4 and β-1,3 glycosidic bonds. It is a major, ubiquitous component of extracellular matrices. The number of repeat disaccharides in a completed hyaluronan matrix 4418 JOURNAL OF BIOLOGICAL CHEMISTRY

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2 The abbreviations used are: DN, diabetic nephropathy; AR, aldose reductase; C/EBP, CCAAT enhancer-binding protein; ED1, mouse monoclonal antibody to CD68; ER, endoplasmic reticulum; GFAT, glutamine:fructose-6-phosphate amidotransferase; GlcUA, glucuronic acid; LC3, microtubule-associated protein 1 light chain 3; 4 MU, 4-methylumbelliferone; 4-MU-xyl, 4-methylumbelliferyl-β-D-xyloside; RMC, rat mesangial cell; TRITC, tetramethylrhodamine isothiocyanate; Dil, 1',1'-dioctadecyl-3,3',3’-tetramethylindocarbocyanine perchlorate.
molecule can reach 20,000 or more, a molecular mass of >8 million Da, and a length of >20 μm. The formation of the monocyte-adhesive hyaluronan matrix by rat renal mesangial cells (RMCs) is a multiphase process that requires the following: 1) a PKC signaling pathway(s) activated in RMCs dividing in hyperglycemic glucose; 2) synthesis and accumulation of hyaluronan in intracellular compartments (8–24 h) that initiates an ER stress/autophagic response, and 3) cyclin D3-mediated formation of the abnormal extracellular monocyte-adhesive hyaluronan matrix after completion of cell division (24–48 h) (10). Understanding these cellular and molecular events will provide significant insights into the mechanisms controlling the extensive influx of macrophages without DN; and 4) heparin has been shown to inhibit mesangial cell growth in our previous studies (25). Some collected kidneys were fixed in 4% paraformaldehyde in PBS at 4 °C overnight for subsequent cryo-embedding and sectioning for histological analyses (Histology Core Facility, Department of Biomedical Engineering, Cleveland Clinic). In parallel, glomeruli were isolated from minced kidneys with a Collector tissue sieve (Belco, San Leandro, CA) as described previously (25).

**Experimental Procedures**

*Reagents*—Streptomyces hyaluronidase, streptococcal hyaluronidase, and chondroitinase ABC were from Seikagaku America Inc. (Rockville, MD). Antibody against cyclin D3 was from BD Biosciences. Antibodies against LC3, macrophage, and C/EBPα were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Thy1.1 monoclonal antibody was from Serotec (Oxford, UK). Anti-rat CD44 monoclonal antibody was from BIOSOURCE. Anti-rat ED1 monoclonal antibody was from AbD Serotec (Raleigh, NC). FITC-heparin and Dil were purchased from Molecular Probes, Invitrogen.

**Establishment of RMC Cultures and Induction of Diabetes in Rats**—RMC cultures were established from isolated glomeruli and characterized as described previously (20, 21). RMCs were used between passages 5 and 15 when they still contract in response to angiotensin II and endothelin, and they exhibit growth suppression in the presence of heparin (1 μg/ml), which are additional characteristics of mesangial cells (22–24). RMCs were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and passaged at confluence by trypsinization for 5 min with a solution of 0.025% trypsin, 0.5 mM EDTA. To render cells quiescent (24), cultures at 40% confluence (2 × 10⁴ cells/cm²) were washed with RPMI 1640 medium and placed in fresh medium containing 0.4% FBS for 48 h (yielding 70–80% confluent cultures).

Hyperglycemic diabetes was induced in ~175-g male Sprague-Dawley rats using tail vein injections of 55 mg/kg streptozotocin as described previously (4, 25). All animals were fed standard laboratory diet. Blood was collected by tail-bleed at day 3 after injection, and the blood glucose concentration was determined by using fluorophore-assisted carbohydrate electrophoresis analyses to confirm the onset of diabetes.

One group of diabetic rats was injected with low molecular weight heparin (Seikagaku, Japan) at 6 mg/kg body weight/day subcutaneously. At 1, 2, 4, and 6 weeks after the onset of diabetes, two rats each from control, diabetic, and diabetic-treated with low molecular weight heparin groups were euthanized by CO₂ asphyxiation, and the kidneys were isolated for immunohistochemistry analysis and isolation of glomeruli as described in our previous studies (25). Some collected kidneys were fixed in 4% paraformaldehyde in PBS at 4 °C overnight for subsequent cryo-embedding and sectioning for histological analyses (Histology Core Facility, Department of Biomedical Engineering, Cleveland Clinic). In parallel, glomeruli were isolated from minced kidneys with a Collector tissue sieve (Belco, San Leandro, CA) as described previously (25).

**Immunohistochemistry**—Cryo-sections of kidneys and methanol-fixed RMC cultures on coverslips were stained for hyaluronan with hyaluronan-binding protein (Seikagaku America) for cyclin D3, LC3, CD44, Thy1.1, and C/EBPα with antibodies and for nuclei with 4,6-diamidino-2-phenylindole, as described previously (9, 10, 26) or according to the manufacturer’s instructions. Samples were treated with biotinylated hyaluronan-binding protein at a 1:100 dilution and with antibodies at a 1:75 dilution, washed, and treated with fluorescein isothiocyanate/streptavidin at 1:500 dilution and/or with antimouse IgG TRITC and anti-rabbit IgG Cy5 antibodies at 1:200 dilution. Stained samples were mounted in VectaShield containing DAPI (Vector Laboratories) for staining the nuclei of cells. Confocal images of the samples were obtained with a Leica TCS-NT laser scanning confocal microscope equipped with four lasers for excitation at 351, 488, 561, and 633 nm wavelengths. The same settings of the confocal microscope and laser scanning were used for both control and treated samples. The magenta signal of Cy5 was converted to green for data presentation using Adobe Photoshop CS2 software from Adobe System (San Jose, CA).
In some experiments, RMC cultures were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and then stained with Nile Red as described previously (27). In other experiments, cryo-sections of kidneys were stained with Oil Red O for assessing lipid accumulation (28). In other experiments, to determine whether or not heparin was internalized by RMCs, growth-arrested cultures were stimulated to divide with 10% FBS in high glucose medium in the presence of 1 μg/ml FITC-heparin for 1, 2, and 4 h. At the end of each incubation period, cells were lightly fixed by 4% paraformaldehyde for 10 min at room temperature and then analyzed by fluorescent microscopy. In some cultures, 0.5 μg/ml DII was added at 30 min before the end of incubation following the manufacturer’s instruction. In the other cultures, cells were treated with FITC-heparin for 2 h and chased in fresh media without FITC-labeled heparin for another 2 h. The images shown represent the results from two different experiments.

Assay for Monocyte Adhesion (9, 29)—RMCs in 6-well plates were treated up to 72 h with 5–20% FBS and concentrations of 5.6 and 25.6 mM D-glucose. Mannitol at 20 mM in 5.6 mM D-glucose was used as an osmotic control. U937 cells were cultured in suspension in RPMI 1640 medium containing 5% FBS and passed at a 1:5 ratio (2 × 10^5 cells/ml) every 48 h (29). Assays for monocyte adhesion were done as described previously (9, 29). After washing, the cell cultures were imaged by microscopy with a Polaroid digital camera (9), and the numbers of monocytes per culture area were counted using ImagePro software. Each culture was equally divided into four regions, and a culture area for imaging was randomly picked in each region. Streptomyces hyaluronidase treatment (1 turbidity reducing unit/ml at 37 °C for 15 min) of RMCs before monocyte incubation was used to determine the extent of the hyaluronan-mediated adhesion.

Fluorophore-assisted Carbohydrate Electrophoresis Analysis of Reducing Saccharides (30, 31)—Cell cultures and isolated glomeruli were incubated with proteinase K at 250 μg/ml in 0.1 M ammonium acetate, pH 7.0, for 3 h at 60 °C (25, 32). The reaction was terminated by heating the samples at 95 °C for 3–5 min. Glycosaminoglycans were recovered by 75% ethanol pre-reaction was terminated by heating the samples at 95 °C for 3–5 min. The digests were dried by centrifugal evaporation. The pellets were dissolved in 0.1 M ammonium acetate, pH 7.0, and incubated with streptococcal hyaluronidase (50 milliunits/ml) and chondroitinase ABC at 2 units/ml overnight at 37 °C to generate disaccharides from hyaluronan and chondroitin/dermatan sulfate. The reaction was terminated by heating the samples at 95 °C for 3–5 min. The digests were dried by centrifugal evaporation and then subjected to reductive amination with 2-aminoacridone as described previously (9). At the end of the incubation, the samples were each mixed with glycerol to 20%, and underivatized aliquots were then subjected to electrophoresis on Glyko Mono Composition gels with Mono Running buffer from ProZyme Inc (San Leandro, CA). Running conditions were 500 V at 4 °C in a cold room for 1 h. Gels were imaged on an Ultra Lum transilluminator (365 nm). Images were captured with a Quantix cooled charge-coupled device camera from Roper Scientific/Photometrics and analyzed with the Gel-Pro Analyzer program version 3.0 (Media Cybernetics). The hyaluronan contents were quantified according to the integrated intensities of signal bands and then normalized with chondroitin contents instead of DNA contents in the samples due to hypercellularity in diabetic glomeruli (4).

RESULTS

Our previous studies (9, 10, 26) showed that growth-arrested (G_{1}/G_{0}) mesangial cells stimulated to divide in hyperglycemic medium activate hyaluronan synthase 2 in intracellular compartments. This initiates hyaluronan synthesis inside these compartments within the cell, which induces ER stress and autophagy. Fig. 1, A and C, show an example of intracellular hyaluronan (green) in permeabilized mesangial cells 16 h after initiating cell division in hyperglycemic (25.5 mM glucose) medium, which is not present in mesangial cells stimulated to divide in normal 5.5 mM glucose (10) (data not shown). The presence of a PKC inhibitor (bisindolylmaleimide I, 100 nM, Fig. 1A) (9), 4-methylumbelliferone (4-MU) (0.2 mM, D) (33) or 4-MU-β-D-xyloside (4-MU-xyl) (0.25 mM, E) (34), or of heparin (2.0 μg/ml, F) prevents the intracellular hyaluronan response of dividing mesangial cells in hyperglycemic medium.

Near the end of cell division a large up-regulation of cyclin D3 was shown to be essential for extrusion of the hyaluronan into a monocyte-adhesive extracellular matrix and also essential for up-regulation of C/EBPα (10). Fig. 2B shows an example of the extensive hyaluronan matrix (green) and cyclin D3-stained aggresomes (red) in permeabilized mesangial cells 48 h after initiating cell division in hyperglycemic medium, which is absent in mesangial cells that divided in normal glucose medium (Fig. 2A). Strikingly, the presence of heparin in the hyperglycemic medium (Fig. 2C) blocked the cyclin D3 aggresome response as well as the intracellular hyaluronan response (Fig. 1), but it still initiated the formation of a much larger hyaluronan matrix (Fig. 2C) as shown in the hyaluronan analyses (Fig. 2F). The presence of the PKC inhibitor (Fig. 2D), 4-MU-xyl (Fig. 2E), or 4-MU (data not shown) in hyperglycemic medium prevented both the cyclin D3-stained aggresomes and the hyaluronan matrix responses.

Fig. 3 shows an example of U937 monocyte adhesion at 4 °C to mesangial cell cultures 48 h after stimulation to divide in hyperglycemic medium (B) or in normal glucose medium as a control (Fig. 3A). The presence of heparin in the hyperglycemic medium greatly increased U937 monocyte adhesion (Fig. 3C) compared with the hyperglycemic medium alone, and digestion of the hyaluronan matrix with Streptomyces hyaluronidase (specific for hyaluronan) prior to adding the U937 monocytes at 4 °C (Fig. 3F) showed monocyte adhesion down to a level equivalent to the normal glucose control. The presence of 4-MU-xyl (Fig. 3E), 4-MU (Fig. 3D), or PKC inhibitor (data not shown) in hyperglycemic medium also prevented U937 monocyte adhesion beyond the level of mesangial cells treated with the normal glucose level control. Quantitation of bound monocytes and hyaluronan in a similar experiment is shown in Fig. 12.

The up-regulation of C/EBPα as a response to mesangial cell division in hyperglycemic medium suggests that lipid synthesis pathways are activated. Fig. 4 shows that this occurs. Nile red staining of 48-h cultures shows extensive lipid contents in mesangial cells that divided in hyperglycemic medium (Fig. 4B) compared with cultures in low glucose (Fig. 4A) and in low
glucose plus mannitol as an osmotic control (Fig. 4).

Furthermore, the presence of heparin prevented this response (Fig. 4), consistent with the absence of up-regulation of cyclin D3 that was necessary for up-regulation of C/EBPα (10).

The mechanisms shown for mesangial cells in vitro occur in vivo in the streptozotocin diabetic rat. Streptozotocin kills pancreatic islet cells, and within 2 days there is complete loss of insulin, and the blood glucose levels are ~25 mM (~5 times normal levels). Fig. 5, A and B, shows glomeruli in sections of kidneys from control and 1-week-old diabetic rats stained for hyaluronan (green) and Thy1.1 (red), a marker for normal mesangial cells. Previous studies have shown that there is extensive division of mesangial cells during the 1st week after streptozotocin treatment (4), and this may reflect the diminished staining for Thy1.1. The abnormal hyaluronan matrix shown at higher magnification (Fig. 5D) appears closely associated with nuclei in several cells (asterisks), consistent with the intracellular accumulation of hyaluronan in dividing hyperglycemic mesangial cells in vitro (Fig. 1) (9, 10, 26). For comparison, hyaluronan staining of a permeabilized 48-h hyperglycemic mesangial cell culture (Fig. 5C) shows examples of hyaluronan matrix closely associated with nuclei (asterisks). Fig. 5F shows the presence of macrophages stained with ED1, a marker for early stage monocytes/macrophages, in a section of a glomerulus from a 1-week-old diabetic kidney (enlarged in insets) and their absence in the glomerulus in a section from a control kidney (Fig. 5E).

Fig. 6B, shows the presence of mesangial cells that underwent autophagy in a section of a glomerulus from a 1-week-old diabetic kidney stained for cyclin D3 (red) and microtubule-associated protein 1 light chain 3 (LC3, green), a marker for autophagy. The glomerulus in the section from a control kidney shows the absence of this process (Fig. 6A). One-week-old diabetic kidney glomeruli also show staining for C/EBPα (Fig. 6D) and lipids (Fig. 6F) that are absent or much less in the normal kidney glomeruli (Fig. 6, C and E). Interestingly, associated renal tubules appear to show similar responses to those in the diabetic glomeruli (Fig. 6, D and F), which suggests that the pathological mechanisms involved may be active in other kidney tissues.

Fig. 7 shows that U937 monocytes adhere at 4 °C in clusters to a section from a 1-week-old diabetic kidney (B and C, reproduced from our review (26)) in contrast to a section from a control kidney (A). Upon warming to room temperature, most of the U937 monocytes were released from the section, collected, spread on a coverslip, and then stained for hyaluronan (Fig. 7D, green) and CD44 (red). The CD44 that is normally uniformly spread on the surface of U937 monocytes formed...
coalesced caps and contained intracellular hyaluronan that was phagocytosed from the section (Fig. 7D). The insets in Fig. 7D show examples of monocytes/macrophages in diabetic kidney sections stained for hyaluronan and CD44, consistent with similar activity possibly occurring in diabetic glomeruli.

When we found that heparin inhibited the autophagy response in mesangial cell cultures while initiating synthesis of an extensive monocyte-adhesive matrix, we initiated a 6-week experiment in which one set of animals, two per time point, received a daily i.p. injection of a heparin preparation consistent with the experimental procedures reported by Gambaro et al. (11, 12), which showed that this prevented nephropathy and proteinuria over an 8-week period in the streptozotocin diabetic rat model. Fig. 8 shows the hyaluronan content normalized to the chondroitin sulfate content of glomeruli at 1, 2, 4, and 6 weeks. As in our previous experiments, the hyaluronan content in the glomeruli from the diabetic rats steadily increased over the 6-week period compared with the unchanged hyaluronan content in the glomeruli from the control rats. In contrast, the hyaluronan in the glomeruli from diabetic rats treated with heparin increased greatly at week 1 compared with both the control and diabetic glomeruli, and then decreased steadily to near the control glomeruli level by week 6. Furthermore, the heparin-treated diabetic animals did not show significant signs of stress, weight loss, or excessive urination typical of the uncontrolled diabetic animals, which is consistent with the results from the Gambaro et al. studies (11, 12).

Fig. 9, A–C, shows the results for glomeruli in 1-week-old kidney sections stained for hyaluronan (green) and LC3 (red). The diabetic glomerulus shows an increase in hyaluronan, consistent with results in Fig. 8, and extensive mesangial cell autophagy compared with the control glomerulus. As also expected from the results in Fig. 8, the hyaluronan content of the glomerulus in the kidney section from the heparin-treated diabetic rat showed an extensive hyaluronan matrix and the absence of any evidence for mesangial cell autophagy, consistent with the results in vitro (Fig. 2). Fig. 9, D–F, shows glomeruli stained for hyaluronan (green) and ED1 (red), a marker for early stage monocytes/macrophages. The glomerulus from the diabetic kidney shows the presence of a significant number of monocytes/macrophages. In contrast, the glomerulus from the kidney in the diabetic rat treated with heparin shows fewer
monocytes/macrophages in the glomerulus, but extensive numbers at the periphery. Fig. 10, A–C, shows glomeruli from the 6-week-old kidneys stained for hyaluronan (green) and Thy 1.1 (red, A, B and D), a marker for normal mesangial cells, or for hyaluronan and ED1 (red, E and F), a marker for monocytes/macrophages (enlarged insets, F). C, shows hyaluronan (green) in a permeabilized hyperglycemic RMC culture that shows close association with nuclei (asterisks) that appear similar to the hyaluronan matrices in the glomerulus section (D) (asterisks) enlarged from B. Nuclei are stained blue.

FIGURE 5. Hyaluronan in sections of glomeruli. Glomeruli (Glm) from control (A and E) and 1-week-old diabetic rats (B, D, and F) were stained for hyaluronan (green) and Thy 1.1 (red, A, B and D), a marker for normal mesangial cells, or for hyaluronan and ED1 (red, E and F), a marker for monocytes/macrophages (enlarged insets, F). C, shows hyaluronan (green) in a permeabilized hyperglycemic RMC culture that shows close association with nuclei (asterisks) that appear similar to the hyaluronan matrices in the glomerulus section (D) (asterisks) enlarged from B. Nuclei are stained blue.

FIGURE 6. Hyaluronan, autophagy, and lipids in sections of glomeruli. Glomeruli (Glm) from control (A, C, and E) and 1-week-old diabetic rats (B, D, and F) were stained for LC3 (green), a marker for autophagy, and cyclin D3 (red, A and B), or for hyaluronan (green) and C/EBPα (red, C and D), or for lipids with Nile red (E and F). Autophagosome structures appear to be present (red) in the enlarged inset (B).

hyperglycemia-induced Hyaluronan Synthesis by Heparin

The results with heparin suggest that the dividing cells may have a receptor that interacts with the heparin and initiates intracellular pathways that prevent the intracellular hyaluronan and autophagy responses. To test this possibility, mesangial cells were stimulated to divide in the presence of FITC-labeled heparin. Fig. 11 shows extensive adherence of the fluorescent heparin to the mesangial cells at 1 h (Fig. 11A), which is internalized and localized in ER and Golgi regions at 2 h (Fig. 11B), as shown by co-localization with Dil (Fig. 11H, Overlay). Some of the heparin is closely associated with nuclei as shown in the enlarged Fig. 11 (D) (asterisks) enlarged from B. Nuclei are stained blue.

FIGURE 6. Hyaluronan, autophagy, and lipids in sections of glomeruli. Glomeruli (Glm) from control (A, C, and E) and 1-week-old diabetic rats (B, D, and F) were stained for LC3 (green), a marker for autophagy, and cyclin D3 (red, A and B), or for hyaluronan (green) and C/EBPα (red, C and D), or for lipids with Nile red (E and F). Autophagosome structures appear to be present (red) in the enlarged inset (B).

The bar graphs show quantitation of the monocyte adhesion and the hyaluronan contents of the cultures. Monocyte adhesion increased ~3- and ~7-fold for the cultures treated with high glucose alone or with heparin, respectively, compared with cultures treated with low glucose. Similarly, hyaluronan contents increased ~1.8- and ~4-fold for these cultures. Importantly, cultures in high glucose exposed to the heparin for only the first 4 h showed nearly the same results as for cultures continuously exposed to heparin. Thus, the signaling pathway(s) initiated by the heparin treatment prevents the PKC-activated intracellular hyaluronan and autophagy responses and greatly increases the hyaluronan matrix after completion of cell division. This correlates with the kinetics of binding and
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FIGURE 7. Adhesion of U937 monocytes to kidney sections. Frozen sections of kidneys from control (A) and 1-week-old diabetic rats (B and C) were incubated at 4 °C with U937 monocytes. Adherence of the monocytes in clusters over glomeruli is apparent (red arrows, enlarged in C). Monocytes released from the sections by incubation at 37 °C were spread on a slide and stained for hyaluronan (green) and CD44 (red). Capping of CD44 and phagocytosis of hyaluronan are apparent (yellow arrowheads in D). Insets show macrophages in sections from a 1-week-old diabetic rat kidney stained for hyaluronan (green) and CD44 (red) for comparison. This figure was used in a review and is reproduced with permission from FEBS (26).

FIGURE 8. Hyaluronan in isolated glomeruli. The graphs show the hyaluronan contents in glomeruli isolated from kidneys in an experiment in which one set of diabetic rats was treated with a daily low dose of heparin, two animals per time point and duplicate analyses for each animal.

internalization of the heparin during the first 4 h after initiating progression of cell division from the G0/G1 interphase. These results also provide strong evidence for a cell surface heparin receptor that would be required to internalize heparin molecules in the 10–20-kDa range and transport them to the ER, Golgi, and nuclear regions.

DISCUSSION

Chronic hyperglycemia in diabetes causes excessive amounts of intracellular glucose and its metabolites, which can activate intracellular metabolic pathways that lead to diabetic complications (35). Four key metabolic pathways of glucose have been studied as potential contributors to hyperglycemia-induced cell damages as follows: 1) increased hexosamine pathway influx; 2) increased polyol pathway influx; 3) activation of protein kinase C (PKC) isoforms; and 4) increased formation of advanced glycation end products. Hyperglycemia-induced mitochondrial reactive oxygen species overproduction has been proposed as a unifying mechanism by inhibiting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity (35). This inhibition would lead to the accumulation of upstream glycolytic metabolites that would be shunted into these four glucose metabolic pathways solely based on the availability of substrates.

The hexosamine pathway is a branch of the glycolytic pathway that utilizes ∼3% of total glucose within cells. In this pathway, fructose 6-phosphate is converted to glucosamine 6-phosphate by the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) (36–38). Subsequent reactions metabolize glucosamine 6-phosphate to the main product, UDP-GlcNAc. Therefore, sustained hyperglycemia would lead to excess UDP-GlcNAc, which is a substrate for hyaluronan synthesis. The amount of glucose diverted into this pathway is controlled by GFAT activity, which is highly regulated at the following: 1) the level of fructose 6-phosphate; 2) feedback inhibition by UDP-GlcNAc via an allosteric mechanism; and 3) the quantity and post-translational phosphorylation of GFAT (36). GFAT is not observed in the normal glomeruli but is expressed in diabetic glomeruli (36). Studies with adipocytes indicate that the half-life of GFAT protein could be less than 1 h (36, 39). Various factors, including EGF, glucose, and glucosamine, regulate GFAT transcriptional expression, and Sp1 GC-boxes have an important role (40). Importantly, the GFAT promoter contains CCAAT-boxes (40, 41), which can be activated by C/EBPα complexed with cyclin D3. Therefore, our data presented here as well as in our previous publications (10), which showed that hyperglycemia induces C/EBPα complexed with cyclin D3, indicate that high glucose could directly regulate GFAT expression via C/EBPα.

Hyperglycemia induces the conversion of ∼30% of intracellular glucose into the polyalcohol sorbitol pathway, whereas only ∼3% is converted under normal physiological conditions (42, 43). This conversion is catalyzed by aldose reductase (AR) with concomitant decreases in NADPH and glutathione, two antioxidants. This is followed by the second oxidation of sorbitol to fructose, which is catalyzed by sorbitol dehydrogenase, which increases NADH. The collective effect of increased NADH/NADPH enhances sensitivity to oxidative stress. In response to high glucose, both renal mesangial and proximal tubule cells accumulate sorbitol (42). Furthermore, increased expression of the AR gene has been observed in response to hyperglycemic glucose in mesangial cell cultures and in experimental animal models (42–44), which could be regulated by an osmotic response element involving PKC activity. Particularly pertinent to our study, the AR promoter also contains a CCAAT-box (45), suggesting that AR can be regulated by C/EBPα, and the product of this pathway, fructose, can enter the hexosamine pathway and provide increases in the substrates for hyaluronan synthesis (46).
As described previously (9), the PKC inhibitor prevents activation of the hyaluronan synthase inside the cell during cell division in hyperglycemic medium, which allows the cell to complete cell division without initiating the autophagy and cyclin D3 responses. In this case, after cell division, the hyperglycemic medium does not initiate increased synthesis of hyaluronan. The results with 4-MU and 4-MU-xyl indicate that elevated concentrations of cytosolic substrates for hyaluronan synthesis, UDP-GlcNAc and UDP-GlcUA, are likely to have a major role for initiating the intracellular hyaluronan synthesis and autophagy responses of the dividing mesangial cells to hyperglycemia. 4-MU, which is often referred to as an inhibitor of hyaluronan synthesis (47–49), is converted to a glucuronide by cells, thereby diminishing UDP-GlcUA (47). However, 4-MU does have side effects such as inducing apoptotic responses (48). 4-MU-xyl is a substrate for chondroitin sulfate synthesis when it enters the Golgi. It increases chondroitin sulfate synthesis almost 10-fold in mesangial cells (34). This
depletes cytosolic UDP-GlcUA and UDP-GalNAc, which must enter the Golgi through antiporters to sustain this highly elevated rate of synthesis of chondroitin sulfate (26). UDP-GlcNAc is the source of cytosolic UDP-GalNAc through a 4-epimerase, and therefore, cytosolic UDP-GlcNAc is also decreased. This diversion of substrates to the Golgi prevents the intracellular hyaluronan synthesis and autophagy responses, and it also prevents the subsequent production of the monocyte-adhesive hyaluronan matrix after completion of cell division. In contrast, although the heparin prevents the responses during cell division in hyperglycemic medium, a cellular mechanism is initiated after cell division is completed that activates synthesis of the much more extensive monocyte-adhesive hyaluronan matrix. This provides an effective cellular response to reduce the continued stress from the sustained hyperglycemic glucose level in the medium.

Activation of protein kinase C by diacylglycerol has been shown in cultured cells and in diabetic glomeruli in response to hyperglycemia, which mediates cellular and tissue damages (50–52). Intracellular hyperglycemia increases de novo synthesis of diacylglycerol in vivo and in vitro due to the increased glycolytic intermediate, dihydroxyacetone phosphate, which is converted to glycerol 3-phosphate by reduction (52, 53). PKCα and PKCβ are the isoforms primarily activated in diabetic glomeruli (50). Activation of PKCα can also regulate nitric oxide...
(NO) synthesis, MAPK activity, and accumulation of extracellular matrix protein (52, 53). PKC is not only required for the expression of cyclin D3, as reported previously (54–56), but also mediates the high glucose-induced synthesis and formation of a monocyte-adhesive hyaluronan matrix in the hyperglycemic RMC cultures (Figs. 1 and 2).

Lipid accumulates in the kidney in diabetic humans and in experimental animal models of diabetes (57–60), and it has been proposed to have a role in the pathogenesis of DN (61, 62). Originally, it was thought that this accumulation is due to increased serum lipids. However, recent studies as well as our own show that there is increased lipid deposition in glomeruli and tubular regions within 1–2 weeks after diabetes onset induced by streptozotocin while maintaining a normal level of serum lipids (60). This lipid accumulation is accompanied by induced expression of sterol regulatory element-binding proteins and fatty-acid synthase, suggesting an increase in renal lipid biosynthesis. In cultured cortical tubule cells and glomerular mesangial cells, elevated expressions of sterol regulatory element-binding proteins and fatty-acid synthase were observed 48 h after high glucose treatment (60), and we observed a significant increase in intracellular lipid deposits in RMC cultures in response to high glucose at 48 h (Fig. 4). Furthermore, in high glucose cultures, Western blots showed large increases in cyclin D3 and C/EBPα at 48–72 h, and a high concentration of a complex with cyclin D3, CDK4, and C/EBPα is already apparent by 24 h (10). High expressions of cyclin D3 and C/EBPα were also prominent in both glomerular and tubular regions in diabetic kidneys 1 and 4 weeks after diabetes onset. Cyclin D3 and C/EBPα are two important mediators during adipocyte differentiation and lipid biosynthesis, and they are expressed in differentiating preadipocytes (63–65). Thus, our data suggest that cyclin D3 and C/EBPα have roles in mediating the lipogenesis induced by hyperglycemia.

A primary response of cells or tissues to hyperglycemia may be to lower glucose levels by an effective mechanism through synthesis of hyaluronan by utilizing intracellular UDP-GlcNAc and UDP-GlcUA. The energy cost for synthesis of a disaccharide of HA is minimal. It requires a single enzyme, and some of the metabolic cost of synthesizing the UDP-sugar precursors is recovered by the production of NADPH from the oxidation of UDP-glucose to UDP-GlcUA, which can be re-oxidized to NADP to yield ATP. It is also now apparent that RMCs dividing in hyperglycemia initiate hyaluronan synthesis inside the cell ~8 h after entering the G1 phase and at or near the entrance to the S phase, which is independent of the normal cell surface activation mechanism (10, 26). However, many cells (RMCs (9, 10), smooth muscle cells (66), and epithelial cells (67)) synthesize a monocyte-adhesive HA matrix in response to various stresses, including ER stress, in normal glucose levels, and our data show that heparin treatment of RMCs dividing in hyperglycemic medium prevents intracellular HA synthesis while still initiating synthesis of an extensive monocyte-adhesive HA matrix. Therefore, future studies can determine the mechanisms of these two distinctly different pathways for producing the abnormal HA matrix. Understanding these pathways will have a major impact on understanding the role of hyperglycemia in diabetic pathologies and in autophagic mechanisms.

The results presented here are consistent with the following model. In the uncontrolled diabetic glomeruli, the mesangial cells that undergo division and autophagy are unable to sustain glomerular function, and the influxed monocytes/macrophages are unable to effectively remove the hyaluronan matrix. This causes a dialogue between the injured mesangial cells and the recruited inflammatory cells that is pro-fibrotic and leads to nephropathy, loss of glomerular function, and proteinurea. In contrast, the mesangial cells in glomeruli from heparin-treated diabetic rats complete cell division without both the intracellular hyaluronan and autophagy responses, and they sustain glomerular function. However, the sustained hyperglycemic stress is compensated by the heparin-induced synthesis of the extensive monocyte-adhesive hyaluronan matrix that rapidly elevates the hyaluronan content in glomeruli by week 1 when monocytes/macrophages are still being recruited. In this case, the dialogue between the mesangial cells and the influx of inflammatory cells supports phagocytic removal of the hyaluronan matrix (similar to the mechanism shown in Fig. 7) without initiating pro-fibrotic responses, which allows the mesangial cells to sustain glomerular function. By 6 weeks the monocyte/macrophage population is sufficient to reduce the hyaluronan matrix being synthesized by the mesangial cells to near control levels (Fig. 8). The results for the experiments with mesangial cells in vitro provide strong evidence to support this model.

Furthermore, our data demonstrate two ways to interfere with the intracellular HA synthesis and the autophagic/cyclin D3 responses as follows: treatment with heparin and with 4-MU-xyl. Although heparin inhibits the PKC signaling pathway(s) that initiates the intracellular HA responses, it initiates an as yet unknown signaling pathway that stimulates formation of a much more extensive monocyte-adhesive HA matrix than occurs in hyperglycemic medium alone. The 4-MU-xyl inhibits both the intracellular responses and the formation of the HA matrix by diverting the cytosolic UDP-sugar substrates for HA synthesis into the Golgi to elevate chondroitin sulfate synthesis (34). Under hyperglycemia, elevated glucose metabolites, UDP-sugars, are major contributors of pathological responses (46). Thus, this study reveals significant new insights regarding the potential therapeutic roles of heparin and its derivatives and of the HA synthesis inhibitor, 4-MU-xyl in DN.

Our previous studies (24, 68, 69) and this study have shown the following: 1) binding of heparin to RMCs is specific, rapid (5–10 min), saturable (within 60 min), and reversible; 2) Scatchard analysis of heparin binding indicates a single class and 6.6 × 10^8 binding sites per cell (K_d = 1.6 × 10^{-8} M) in quiescent cells; 3) surface-bound heparin can be internalized and degraded; 4) the affinity and number of heparin-binding sites are affected by the stage of RMC growth; and 5) heparin acts at the RMC surface to affect both PKC-dependent and -independent pathways. By examining the antiproliferative effect of heparin on RMCs, our previous study suggests that even at concentrations below 1 μg/ml at least two mechanisms contribute, one operating very early after stimulation (within 15 min) before c-fos expression, and the other relatively insensitive to the timing of events in early progression through G1. This study showed that the rapid internalization of heparin by RMCs...
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was observed in perinuclear regions or ER within 2 h after initiating cell division, and that short treatment of RMCs with heparin for 4 h is sufficient to induce the monocyte-adhesive HA matrix formation after completion of cell division. The mesangial cells in this study were growth-arrested in the G_0/G_1 phase. They re-enter the G_1 phase of cycle within 30 min after serum stimulation and progress into S phase at 12 h. These results clearly indicate that the interaction between heparin and mesangial cells at the early G_1 phase of the cell cycle has an essential role in regulating the formation of the hyaluronan matrix induced by heparin under the hyperglycemic condition at the end of cell division.

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