Hyaluronan Structures Synthesized by Rat Mesangial Cells in Response to Hyperglycemia Induce Monocyte Adhesion*

Received for publication, November 3, 2003, and in revised form, December 16, 2003
Published, JBC Papers in Press, December 16, 2003, DOI 10.1074/jbc.M312045200

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Mesangial expansion, the principal glomerular lesion in diabetic nephropathy, is preceded by a phenotypic activation and transient proliferation of the glomerular mesangial cells and by a prominent glomerular infiltration of monocytes and macrophages. Because this infiltration seems to play a key role in the subsequent mesangial matrix expansion, we tested the response of cultures of rat mesangial cells (RMCs) for monocyte adhesion in response to hyperglycemia. Increasing the medium glucose concentration from 5.6 mM (normal) to 25.6 mM (hyperglycemic) significantly increased hyaluronan in the cell matrix, with a concurrent 3- to 4-fold increase in adhesion of U937 monocyte-like cells to cultures of near confluent RMCs. These responses were attributed directly to the high glucose concentration and not to increased extracellular osmolality. The monocytes primarily bind directly to hyaluronan-based structures in vitro. Abnormal deposits of hyaluronan were found in glomeruli of kidney sections from diabetic rats 1 week after streptozotocin treatment, often with closely associated monocytes/macrophages, suggesting that similar structures are relevant in vivo. The monocyte adhesion response to high glucose concentration required growth stimulation of RMCs by serum and activation of protein kinase C, and was inhibited by prior passage of the RMCs in the presence of heparin. These results suggest that the response may be cell growth state and protein kinase C-dependent. When incubated with the viral mimic poly I:C, in the presence of normal glucose, heparin-passaged RMCs still increased cell-associated hyaluronan and exhibited hyaluronan-mediated adhesion of monocytes, indicating that the two stimuli, high glucose and viral mimetic, induce the production of the hyaluronan structures that promote monocyte adhesion by distinctly different intracellular signaling mechanisms.

Mesangial expansion, the principal glomerular lesion in diabetic nephropathy, reduces the area for filtration and leads eventually to sclerosis and renal failure (1, 2). However, the expansion of the mesangial extracellular matrix (ECM) is not the sclerosis that characterize diabetic nephropathy are preceded by a phenotypic activation and transient proliferation of the glomerular mesangial cells. This is followed by a prominent glomerular infiltration of monocytes and macrophages (3–5) that seems to play a key role in the subsequent mesangial matrix expansion, hypercellularity, and onset of proteinuria (6, 7). Nevertheless, the molecular mechanisms underlying glomerular infiltration by monocytes and macrophages are still unclear.

The interaction of monocytes with mesangial cells in culture has been studied previously using U937 cells, a monocytic leukemic cell line that is widely accepted as a model for monocyte adhesion (7, 8), and mesangial cells cultured in media with high glucose concentrations bind more U937 cells (7). Generally, the interactions between monocytes and resident cells involve: (i) monocyte cell surface proteins, including CD44, β2 leukocyte integrins (LFA-1, CR3, and p150/95), the β1 integrin VLA-4, and L-selectin, and (ii) their ligands, such as VCAM-1 and ICAM-1, on resident cells (9, 10).

Previously, we showed that monocytes preferentially bind, by means of a mechanism that involves CD44, to hyaluronan cube-like structures synthesized by smooth muscle cells isolated from human intestinal mucosa, after they have been treated with a virus or with the viral mimetic, poly I:C (a synthetic double-stranded RNA) (11, 12). This indicates that structures based on hyaluronan can mediate the adhesion of monocytes to resident cells. Previous studies have shown increased hyaluronan content in glomeruli isolated from diabetic animals (13) and in mesangial cells cultured in media with a high glucose concentration (14). Exposure to high glucose concentrations also increases hyaluronan synthesis in renal proximal tubular cells (15), in renal interstitial fibroblasts (16), and in vascular smooth muscle cells (17), all of which are involved in normal kidney physiology.

Experiments described in this report indicate that: (i) cultures of rat mesangial cells (RMCs) exposed to high glucose concentrations can produce hyaluronan structures that promote monocyte adhesion; (ii) this process depends on the growth state and protein kinase C (PKC) activation of the RMCs; (iii) hyaluronan deposits, often with closely associated monocytes/macrophages, are apparent in 1-week diabetic rat glomeruli; and (iv) RMCs passaged in the presence of heparin lose their ability to produce the adhesive hyaluronan structures in response to high glucose concentrations but continue to do so when incubated with the viral mimetic, poly I:C, in medium with normal glucose concentration. These results support the hypothesis that hyaluronan structures in the mesangial ECM, produced in response to hyperglycemia, bind to specific receptors on the monocyte surface, thereby promoting

* This work was supported by National Institutes of Health Grant KO1 DK02847 (to A. W.) and Diabetes Association of Greater Cleveland Grant 453 (to A. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: ECM, mesangial extracellular matrix; PKC, protein kinase C; RMCs, rat mesangial cells; PBS, fetal bovine serum; FACE, fluorophore-assisted carbohydrate electrophoresis; PBS, phosphate-buffered saline; poly I:C, a synthetic double-stranded RNA; HBP, hyaluronan-binding protein; DAPI, 4',6-diamidino-2-phenylindole; TRITC, tetramethylrhodamine isothiocyanate.

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monocyte and macrophage adhesion to mesangial matrix in the early stages of diabetic nephropathy.

**EXPERIMENTAL PROCEDURES**

**Establishment of RMC Cultures and Induction of Diabetes in Rats—**RMC cultures were established from isolated glomeruli and characterized as described previously (18, 19). 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 (20–22). RMCs were cultured in RPMI 1640 medium containing 10% 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 (22), cultures at ~40% confluence (~2 × 10^5 cells/cm^2) were washed with RPMI 1640 medium and placed in fresh medium containing 0.4% FBS for 48–72 h (yielding 70–80% confluent cultures). To prepare the heparin-pretreated cells, the RMCs were cultured for three or five passages in the presence of 10 µg/ml heparin (23). U937 cells were cultured in suspension in RPMI 1640 medium containing 5% FBS and passaged at a 1:5 ratio (~2 × 10^6 cells/ml) every 48 h (11).

Diabetes was induced in ~175-g male Sprague-Dawley rats using tail vein injections of 55 mg/kg streptozotocin (5, 24). All animals were fed standard laboratory diet. Blood was collected by tail-bleeding at day 3 after injection, and the blood glucose concentration was determined by using fluorophore-assisted carbohydrate electrophoresis (FACE) analyses to confirm the onset of diabetes. After 1 week, the kidneys were collected from both control and diabetic rats and fixed in 4% paraformaldehyde in PBS at 4 °C overnight for subsequent processing and sectioning for histological analyses (Histology Core Facility, Department of Biomedical Engineering, Cleveland Clinic Foundation).

**Immunohistochemistry—**Thin sections of paraffin-embedded kidneys and methanol-fixed RMC cultures on coverslips were stained for hyaluronan with a hyaluronan-binding protein (HABP) (Seikagaku America), for nuclei with 4',6-diamidino-2-phenylindole (DAPI), and in the sections, for monocytes/macrophages with anti-rat ED1 antibody, as described previously (28, 29). Samples were treated with HABP at a 1:100 dilution and with anti-rat ED1 antibody at a 1:75 dilution, for nuclei with 4',6-diamidino-2-phenylindole (DAPI), and in the sections, for monocytes/macrophages with anti-rat ED1 antibody, as described previously (28, 29).

**Proteinase K Release of Glycosaminoglycans from Cells and Tissues (28–30)—**Cell cultures were incubated with proteinase K at 250 µg/ml in 0.1 M ammonium acetate, pH 7.0, for 3 h at 60 °C. The reaction was terminated by heating the samples at >95 °C for 3–5 min.

**Hyaluronidase and Chondroitinase ABC Digestion—**Samples were incubated with streptococcal hyaluronidase (50 milliunit/ml) and chondroitinase ABC at 2 units/ml in 0.1 M ammonium acetate, pH 7.0, 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.

**FACE Analysis of Reducing Saccharides—**Sample digests were dried by lyophilization in microtubes and then subjected to reductive amination with 2-aminooxazole (31). The end of the incubation, the samples were each mixed with glycerol to 20%; 5-µl aliquots were then subjected to electrophoresis on Mono-Composition gels with Mono Running buffer (Glyko). Running conditions were 500 V at 4 °C in a cold room for 1 h. Gels were imaged on an \[ \text{Figure 1. FACE analysis of hyaluronan contents in mesangial cell cultures. A, serum-starved RMCs in 6-well plates were stimulated with 10% FBS in the presence of 5.6–25.6 mM glucose for 72 h. After washing with PBS twice, they were then treated with proteinase K to release glycosaminoglycans, and the hyaluronan contents were determined by FACE analyses. Images were captured with a Quantix cooled charge-coupled device camera. The mean values and standard errors were calculated from two replicate experiments (**, p < 0.01; n = 6). The disaccharide bands derived from chondroitin sulfate are unsulfated (C6S), 4-sulfated (C4S), or 6-sulfated (C0S). The bands identified by the asterisk are glucose, maltose, maltotriose, and maltotetraose derived from inside the cells. B, the fluorescence intensities of target bands were quantified using the Gel-Pro Analyzer version 3.0 program.}

**RESULTS**

**Glucose-induced Hyaluronan Synthesis in RMCs—**When pre-confluent RMC cultures were incubated in 0.4% serum for 48 h, ~75% of the starred cells were arrested in G0/G1 phase, as shown previously by using flow cytometry, and ~72% subsequently progress to S-phase by 18 h after serum stimulation (31). Therefore, after 48 h of serum-starvation, quiescent RMCs re-enter the cell cycle with good synchrony upon stimulation. Triplicate cultures of quiescent, near-confluent, and serum-starved RMC cultures were incubated for 72 h with 10% FBS in the presence of different concentrations of glucose, or in 5.6 mM glucose with 20 mM mannitol as an osmotic control. The hyaluronan contents of the cell layers were assessed by FACE analysis as described under “Experimental Procedures.” Quantification of the hyaluronan disaccharide in FACE gels such as that shown in Fig. 1 indicates that glucose treatment causes a statistically significant increase in hyaluronan content in the cell layers for the 20.6 and 25.6 mM glucose cultures and a trend for an increase between 5.6 and 15.6 mM glucose cultures.
Further, the synthesized hyaluronan induced by high (25.6 mM) glucose was formed into large cable-like structures in RMC cultures. The serum-starved mesangial cells were incubated 72 h with medium containing 10% FBS in the presence of normal (5.6 mM, panel A) or high (25.6 mM, panels C and D) glucose. Mannitol (20 mM) with 5.6 mM glucose was used as an osmotic control (B). Hyaluronan was stained with HABP (green), and nuclei were stained with DAPI (blue). There are large hyaluronan structures in the cultures treated with high glucose (panels A–C, ×40; panel D, ×63).

Fig. 2. Confocal microscopic analysis of hyaluronan structures in RMC cultures. The serum-starved mesangial cells on coverslips were incubated 72 h with medium containing 10% FBS in the presence of normal (5.6 mM, panel A) or high (25.6 mM, panels C and D) glucose. Mannitol (20 mM) with 5.6 mM glucose was used as an osmotic control (B). Hyaluronan was stained with HABP (green), and nuclei were stained with DAPI (blue). There are large hyaluronan structures in the cultures treated with high glucose (panels A–C, ×40; panel D, ×63).

Effect of Glucose Concentration on Monocyte Adhesion to Rat Mesangial Cells—In the experiment in Fig. 3, near-confluent and serum-starved RMC cultures were incubated for 72 h in medium containing 10% FBS or in 5.6 mM glucose with 20 mM mannitol. Monocyte adhesion was then assayed using the U937 monocytic leukemia cell line, as described previously (11). Monocyte adhesion to RMCs incubated with 25.6 mM glucose (Fig. 3B) was dramatically increased compared with RMCs treated with 5.6 mM glucose (Fig. 3A) or with 20 mM mannitol in 5.6 mM glucose as an osmotic control (Fig. 3C). The presence of serum (0.5%) showed monocyte adhesion similar to the 5.6 mM glucose cultures (data not shown). The role of hyaluronan in the adhesion process was underscored by treatment of RMCs incubated with high glucose with testicular hyaluronidase before assessing monocyte adhesion (Fig. 3D), in which case monocyte adhesion was greatly diminished.

At a higher magnification (Fig. 4A, arrow), the adhesive monocytes in the RMCs incubated with high glucose were often arrayed in bead-like strands that appear similar to the patterns observed for monocyte adhesion to hyaluronan cable structures synthesized by colon smooth muscle cells in response to viral stimuli (11, 12). They also often appear in clusters (Fig. 4A, arrowhead). Confocal microscopic analysis of high glucose-treated cultures stained for hyaluronan (green) and nuclei (blue) (Fig. 4, B and C) show these configurations more clearly. In each case, the monocyte nuclei (Fig. 4, arrowheads) are closely arrayed to hyaluronan adhesive structures.

In separate experiments, we have shown that glomeruli isolated from diabetic rats 1 week after treatment with streptozotocin have 2–3 times as much hyaluronan as glomeruli from control rats using the FACE procedures.2 This result would be

2 M. Lauer, V. C. Hascall, A. Wang, unpublished data.
increased with exposure to increasing amounts of glucose, with the number of adherent monocytes per unit area being determined as described above (Figs. 3 and 5). Conversely, RMC cultures passed in the presence of heparin no longer responded to the high glucose treatment, neither in increased hyaluronan production in the cell layer nor in increased monocyte adhesion (Figs. 6 and 7). However, these heparin-passaged RMCs still increased cell-associated hyaluronan and exhibited hyaluronan-mediated monocyte adhesion when incubated with the viral mimetic, poly I:C, in the presence of heparin sulfate (5). Fig. 4 shows kidney tissue sections from diabetic glomeruli (5). The cell cultures were imaged by microscopy with a Polaroid digital camera, and the numbers of monocytes per culture area were counted using MetaMorph software.

At 25.6 mM, there were more than 3 times as many adherent monocytes as observed with 5.6 mM glucose, and ~75% of the difference was lost when RMC cultures exposed to 25.6 mM glucose were pretreated with hyaluronidase. Further, there is a correlation between increased hyaluronan content and increased adhesion of monocytes.

The mannitol osmolarity control showed no difference from the normal (5.6 mM) glucose cultures. Further, pretreatment of RMC cultures exposed to normal (5.6 mM) glucose with hyaluronidase did not significantly alter the number of adherent monocytes (data not shown). Interestingly, the amount of hyaluronan in the cell layer of the RMC culture exposed to normal (5.6 mM) glucose was about half of that of the 20.6 and 25.6 mM glucose cultures (Fig. 1), yet hyaluronan-mediated adhesion in the 5.6 mM glucose cultures was negligible. Thus, monocytes adhering to RMC cultures treated with normal glucose concentration did so by a mechanism independent of hyaluronan, possibly by means of a VCAM-1/VLA-4 mechanism (11).

The hyaluronan-based monocyte adhesion response described above occurs when quiescent preconfluent RMCs are stimulated to enter a mitotic cycle by replenishing serum, but in the presence of a high glucose concentration. In contrast, when confluent RMC cultures were treated identically, the number of adherent monocytes in cultures treated with 25.6 mM glucose did not increase significantly beyond the number adhering to preconfluent cultures in response to 5.6 mM glucose (Fig. 5C). This result suggests that the response is likely to be cell growth state dependent.

Effects of Heparin on Glucose-induced Monocyte Adhesion to RMCs—Diabetic nephropathy in animal models is ameliorated when the animals are treated with heparin or heparan sulfate in vivo (32, 33). Therefore, two different isolates of RMCs were passaged either 3 or 5 times in the presence or absence of 10 µg/ml heparin. Near-confluent RMC cultures were then prepared and exposed to 25.6 mM glucose in the presence of 10% serum and in the absence of heparin. In both isolates, RMCs passaged without heparin showed increased hyaluronan and adhesion of monocytes in response to high glucose treatment (Figs. 6 and 7, CON), as described above (Figs. 3 and 5). Conversely, RMCs passaged in the presence of heparin no longer responded to the high glucose treatment, neither in increased hyaluronan production in the cell layer nor in increased monocyte adhesion (Figs. 6 and 7). However, these heparin-passaged RMCs still increased cell-associated hyaluronan and exhibited hyaluronan-mediated monocyte adhesion when incubated with the viral mimic poly I:C, in the presence of normal (5.6 mM) glucose (Figs. 6 and 7). RMCs passaged without heparin also produced hyaluronan and promoted monocyte adhesion when exposed to poly I:C in the same conditions (data not shown). These results indicate that: (i) RMCs undergo phenotypic changes that make them unresponsive to high glucose when passaged in the presence of heparin; and (ii) there are two completely different mechanisms (high glucose and viral stimulus) for inducing the production of the hyaluronan structures that promote monocyte adhesion.

High glucose increases PKC activity and alters subcellular localization of specific PKC isoforms in mesangial cell cultures (34–38). Heparin pre-treatment of smooth muscle cells decreases the total PKC activity by 50% (23, 39). Thus, the observation that pre-treatment of rat mesangial cells with heparin inhibited glucose-induced monocyte adhesion to cultured mesangial cells indicates that activation of PKC by high glucose might be required for the formation of monocyte adhesive hyaluronan structures. When RMCs were cultured in media containing specific PKC inhibitors, bisindolylmaleimide I or Gö 6976, the high glucose-induced increases in hyaluronan and in
monocyte adhesion to cultured mesangial cells were dramatically inhibited (Figs. 8 and 9). These results strongly suggest that the intracellular PKC signaling pathway is a major mediator in increased hyaluronan synthesis and in monocyte adhesion to cultured mesangial cells in response to high glucose.

**DISCUSSION**

Hyaluronan is a linear glycosaminoglycan composed of repeating disaccharide units of N-acetylgalactosamine and D-glucuronic acid; it consists of alternating β-1,4 and β-1,3 glycosidic bonds and is synthesized at the inner side of the plasma membrane by hyaluronan synthase enzymes. The growing chain is extruded directly into the extracellular space by hyaluronan synthase enzymes. The growing chain is extruded directly into the extracellular space (40–42). These complex, cell-associated structures have important roles in cell proliferation and migration by destabilizing cell adhesion and facilitating changes in cell shape (40–42).

Previous studies have shown that high glucose treatment of mesangial cell cultures induced monocyte adhesion (7), and our studies now show that about 75% of the glucose-induced adhesion of monocytes is actually mediated by hyaluronan structures. The organization of these unique structures must involve hyaluronan-binding proteins (hyaladherins), such as versican and the protein product of the TNF-α-stimulated gene 6, as well as the serum-protein complex, inter-α-trypsin inhibitor (25). No doubt, the different organizations of hyaluronan with cell surface and ECM hyaladherins define different functions of cells in normal and pathogenic processes. Our data in the present study indicate that these adhesive hyaluronan structures in the mesangial ECM produced in response to hyperglycemia likely mediate the adhesion of monocytes in vivo as well as in vitro.

The genesis phase of diabetic nephropathy in experimental animal models involves an early, transient proliferative response of mesangial cells just before monocyte/macrophage infiltration into glomeruli (3–5). Our ongoing studies with streptozotocin-induced diabetes in rats have shown that the hyaluronan content of glomeruli increases 2- to 3-fold within a week after onset of the treatment. Further, we now show an abnormal accumulation of hyaluronan in the glomeruli of diabetic rats, and glomerular infiltrated monocyte/macrophages were embedded in this hyaluronan enriched matrix (Fig. 4).

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3 See also the mini-review series on hyaluronan in the February 15, 2000 issue of the *Journal of Biological Chemistry.*
Thus, our present results support the concept that, in response to hyperglycemia, mesangial cells produce a hyaluronan-based matrix during their proliferative phase that has a structure recognized by inflammatory cells, which then adhere and undergo activation. If correct, this novel matrix structure has an essential role in the subsequent development of the diabetic nephropathy.

Other early events in response to hyperglycemia are significant increases in TGF-β and prostaglandins within the first week (34, 43–47). These factors may be responsible for inhibiting further mesangial cell proliferation and may also be involved in inducing the production of the hyaluronan matrix. High glucose increases PKC activity and alters subcellular localization of specific PKC isoforms in mesangial cell cultures and in glomeruli isolated from diabetic animals by increased de novo synthesis of diacylglycerol from glycolytic intermediates (14, 34–38, 48). PKC activation triggers mitogen-activated protein kinase, which (i) up-regulates TGF-β, and (ii) activates phospholipase A2, thereby increasing production of cellular arachidonic acid and subsequently increasing prostaglandin production. These results suggest that the PKC signaling pathway may be a major mediator in the response of mesangial cells to hyperglycemia. Further, heparin pre-treatment of smooth muscle cells decreases the total PKC activity by 50% (23, 39), and we observed that passage of rat mesangial cells in the presence of heparin inhibited their subsequent ability to synthesize the adhesive hyaluronan structures in response to high glucose concentration. Heparin treatment of smooth muscle cells has also been shown to inhibit MeK, an intermediate in both of the PKC and mitogen-activated protein kinase pathways (23). This could be a key, therefore, to understanding both our observation that exposure of RMCs to low levels of heparin during passage makes them refractory to the high glucose response and the fact that heparin treatment in vivo blunts the diabetic nephropathy response (32, 33). Indeed, the specific PKC inhibitors, bisindolylmaleimide I and Go 6976, dramatically inhibited the high glucose-induced monocyte adhesion to cultured rat mesangial cells (***, p < 0.001; n ≥ 9).

RMCs passaged in heparin still produce adhesive hyaluronan structures in normal glucose concentration when exposed to the viral mimic poly I:C. Thus, the response of these cells, as well as smooth muscle cells (11), to a pathogen, such as a virus, leads to the same or nearly the same outcome but through a distinctly different intracellular signaling pathway. This suggests that different cellular responses have evolved to produce a matrix with information that elicits a host defense response by engaging and activating inflammatory cells. Recent studies (49) have shown that one mechanism for initiating the hyaluronan adhesive structures is the cell response to endoplasmic reticulum stress. The fact that the inflammatory
cells do not respond to the presence of hyaluronan in the “normal” matrix produced by the mesangial cells in normal glucose concentrations indicates that other components organized in the adhesive structures are involved in the adhesive mechanism by the monocytes. The observation that CD-44 on the monocytes/macrophages is involved in the process (11) does, however, indicate that direct interaction with hyaluronan is a necessary part of the mechanism.

Finally, exposure to high glucose concentrations also increases hyaluronan synthesis in renal proximal tubular cells (15), in renal interstitial fibroblasts (16), and in vascular smooth muscle cells (17). Thus, the broader implications of this model for diabetic pathology become clearer. A similar abnormal hyaluronan-based structure may be central to the other complications in diabetes, including inflammatory responses in micro- and macro-vascular diseases.

Acknowledgment—We especially thank Dr. Carol de la Motte for her technical help and suggestions.

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