Mannose Inhibits Hyaluronan Synthesis by Down-regulation of the Cellular Pool of UDP-N-acetylhexosamines

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We found that D-mannose dose-dependently decreases hyaluronan synthesis in cultured epidermal keratinocytes to ~50%, whereas glucose, galactose, and fructose up to 20 mM concentration had no effect. The full inhibition occurred within 3 h following introduction of mannose and did not involve down-regulation of hyaluronan synthase (Has1–3) mRNA. Following introduction of mannose, there was an ~50% reduction in the cellular concentration of UDP-N-acetylhexosamines (UDP-HexNAc, i.e. UDP-N-acetylglucosamine and UDP-N-acetyl-β-galactosamine). On the other hand, 2 mM glucosamine in the introduction of mannose, there was an increase of cellular concentration of UDP-glucuronic acid, the other building block for hyaluronan synthase, was not reduced by mannose content. The content of UDP-glucuronic acid, the other building block for hyaluronan synthesis, was not reduced by mannose but declined from 39 to 14% of controls by 0.2–1.0 mM 4-methylumbelliferone, another compound that inhibits hyaluronan synthesis. Applying 4-methylumbelliferone and mannose together produced the expected reductions in both UDP sugars but no additive reduction in hyaluronan production, indicating that the concentration of each substrate alone can limit hyaluronan synthesis. Mannose is a potentially useful tool in studies on hyaluronan-dependent cell functions, as demonstrated by reduced rates of keratinocyte proliferation and migration, functions known to depend on hyaluronan synthesis.

Hyaluronan is a ubiquitous glycosaminoglycan present in variable concentrations in the pericellular and extracellular matrix of vertebrate tissues. The general functions most frequently associated with hyaluronan are extracellular matrix organization (1) and adhesion, migration and proliferation of cells (2, 3), processes particularly important in fetal development (4), wound healing (5), inflammation (6, 7), and cancer (8–13). Hyaluronan as a hydrophilic, viscous polysaccharide increases the volume of the extracellular space, thus contributing to tissue remodeling and facilitating cell mobility (1). This classical view of hyaluronan as a space filler and swelling agent has been complemented more recently by interesting findings indicating that it mediates homing of stem cells (14), leukocyte adherence to endothelial cells (15), monocyte binding to virally infected (16) or stressed (17) mesenchymal cells, and bacterial adherence (18). Furthermore, high molecular mass hyaluronan and its oligosaccharide degradation products create specific intracellular signals that promote cell locomotion (19, 20), influence cell division (21, 22), block apoptosis, and induce membrane transporters (23). The expanding list of cellular and molecular processes in which hyaluronan is involved has resulted in a growing interest in its metabolism, including possible medical applications.

Hyaluronan synthases (HAS1–3), 2 a family of vertebrate enzymes producing hyaluronan, are inserted in the plasma membrane and deliver the growing glycosaminoglycan directly into the extracellular space. The enzymes utilize cytosolic UDP-glucuronic acid (UDP-GlcUA) and UDP-N-acetylglucosamine (UDP-GlcNAc) as donors to produce the linear hyaluronan polymer containing up to 25,000 repeating disaccharides with the structure [GlcUAβ1→3GlcNAcβ1→4]n (24). Hyaluronan synthesis is known to be controlled at the level of Has transcription (25), which is influenced by growth factors, cytokines, and hormones (2, 3, 25–30). There is also evidence for post-transcriptional factors like HAS protein phosphorylation that may contribute to the rate of hyaluronan synthesis (31, 32). The expression of UDP-glucose (UDP-Glc) dehydrogenase (UGDH), which converts UDP-Glc to UDP-GlcUA, is often up-regulated at the same time as hyaluronan production increases (33), and experimental increase and decrease of UGDH expression exert corresponding changes in hyaluronan synthesis (34). This suggests that the rate of hyaluronan synthesis may also be regulated by the level of HAS substrate concentration. Indeed, 4-methylumbelliferone (4-MU) as a good substrate of glucuronidation consumes UDP-GlcUA and inhibits hyaluronan synthesis (35).

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** The abbreviations used are: HAS, hyaluronan synthase; 4-MU, 4-methylumbelliferone; ELSA, enzyme-linked sorbent assay; GlcN-1P, glucosamine 1-phosphate; GlcN-6P, glucosamine 6-phosphate; GFAT, glutamine-fructose-6-phosphate amidotransferases; GPI, glucosamine-6-phosphate isomerase; HABC, hyaluronan binding complex of the cartilage aggrecan G1 domain and link protein; bHABC, biotinylated HABC; PBS, phosphate-buffered saline; REK, rat epidermal keratinocytes; UGDH, UDP-glucose dehydrogenase; UDP-GlcUA, UDP-glucuronic acid; RT, reverse transcription; MS/MS, tandem mass spectrometry; HPLC, high pressure liquid chromatography; AppCp, β-γ-methylene adenine 5′-triphosphate.

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7666 JOURNAL OF BIOLOGICAL CHEMISTRY
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The pool of UDP-HexNAc is generally the largest among the cellular nucleotide sugars. For example, UDP-GlcNAc is 3–7 times more abundant than that of UDP-GlcUA (36). There is an equilibrium between UDP-GlcNAc and UDP-N-acetylglactosamine (UDP-GalNAc), mediated by UDP-galactose 4-epimerase (37), resulting in UDP-GlcNAc/UDP-GalNAc ratio of ~3:1 (36, 38). Hexosamine biosynthesis is started by glutamine:fructose-6-phosphate amidotransferases (GFAT1 and GFAT2), which are considered as the rate-limiting enzymes in this pathway, and use glutamine and fructose 6-phosphate as substrates to make glucosamine 6-phosphate (GlcN-6P). GlcN-6P is further converted to glucosamine 1-phosphate (GlcN-1P), acetylated, and used for the production of UDP-GlcNAc. An estimated 2% of total intracellular glucose flux goes to hexosamine biosynthesis (39). The hexosamine biosynthesis pathway has received wide interest, because it may act as a cellular glucose sensor and control cellular energy metabolism, being associated with insulin resistance and diabetes (39). However, the influences of cellular UDP-HexNAc fluctuations on glycoconjugates have received less attention (40, 41), probably because it has been assumed that the relatively high concentrations of the nucleotide amino sugars are not rate-limiting in their biosynthesis.

In this study we present evidence that despite its relatively high cellular content, the UDP-HexNAc level influences the synthesis of hyaluronan, and that depletion of either UDP-GlcNAc or UDP-GlcUA alone can limit the rate of hyaluronan synthesis, and that mannose through its ability to decrease UDP-HexNAc is a novel, potentially useful inhibitor of hyaluronan production.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Rat epidermal keratinocytes (REKs) (42) were grown in minimum essential medium, low glucose (Euroclone®, Milan, Italy) supplemented with 5% fetal bovine serum (HyClone, Logan, UT), 4 mM l-glutamine (Euroclone), 50 units/ml penicillin, and 50 μg/ml streptomycin (Euroclone) at 37 °C. Cells were passaged twice a week at 1:5 split ratio using 0.05% trypsin, 0.2% EDTA in phosphate-buffered saline (PBS) (Bichrom AG, Berlin, Germany). For biochemical assays cells were grown close to confluency in 6-, 12- or 24-well plates and then changed to Dulbecco’s modified Eagle’s medium, without glucose (Invitrogen) supplemented with 5% serum, L-glutamine and 5 mM glucose. For biochemical assays cells were grown close to confluency in 6-, 12- or 24-well plates and then changed to Dulbecco’s modified Eagle’s medium, without glucose (Invitrogen) supplemented with 5% serum, l-glutamine, the antibiotics, and 5 mM glucose. This basic control medium was supplied with 20 mM final concentration of galactose, glucose, and fructose, 2 mM glucosamine, and different concentrations of mannose (all purchased from Sigma), as indicated in each experiment.

**Enzyme-linked Sorbent Assay (ELSA) for Hyaluronan**—Approximately 60,000 REK cells were plated on 24-well plates and cultured for 2 days. The medium was then changed, and appropriate concentrations of hexoses were added, followed by 6- or 24-h incubations. Hyaluronan content in the culture medium was measured with a sandwich-type ELSA, as described (11), and cells were released with trypsin-EDTA and counted using a hemocytometer. Ninety six-well Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 1 μg/ml of the hyaluronan-binding complex of the cartilage aggrecan G1 domain and link protein (HABC). HABC was prepared in our laboratory from bovine articular cartilage as described previously (43). Hyaluronan standards (1–50 ng/ml) and samples diluted into 1% bovine serum albumin in PBS were incubated in the wells for 1 h at 37 °C. After washes, the wells were sequentially incubated with 1 μg/ml biotinylated HABC (hHABC) and horseradish peroxidase/streptavidin (1:20,000 in PBS, Vector Laboratories) for 1 h each at 37 °C, followed by 10 min of incubation at room temperature with the TMB substrate solution (0.01% 3,3’,5,5’-tetramethylbenzidine (Sigma) and 0.005% H2O2 in 0.1 mM sodium acetate, 1.5 mM citric acid buffer). The reaction was stopped with 50 μl of 2 M H2SO4, and the absorbances were measured at 450 nm.

**Histochemical Demonstration of Hyaluronan**—The cells cultured on 8-well chamber slides (Nalge Nunc, Naperville, IL) were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 20 min at room temperature and permeabilized with 0.1% Triton X-100 in 1% bovine serum albumin-phosphate buffer for 10 min. The cells were then incubated with bHABC (3 μg/ml) overnight at 4 °C (43), followed by a 1-h incubation with avidin-biotin peroxidase (1:200 ABC-standard kit, Vector Laboratories), and a 5-min incubation in 0.05% 3,3’-diaminobenzidine and 0.03% hydrogen peroxide. The cells were mounted using Supermount (BioGenex, San Ramon, CA). The specificity of the hyaluronan staining was confirmed by pretreatment with Streptomyces hyalurondiase (Seikagaku Kogyo Co., Tokyo, Japan).

**Quantitative RT-PCR**—For RT-PCR, total RNA was isolated from monolayer REK cultures with EUROzol (Euroclone). The samples were DNase-treated with TURBO DNA-free™ (Ambion, Austin, TX), quantitated with a spectrophotometer, and equal amounts of RNA were taken for the reverse transcriptase reaction. Quantitative PCR was performed in a MX3000P thermal cycler (Stratagene, La Jolla, CA), with Absolute™ MAX two-step quantitative RT-PCR SYBR® Green kit (ABgene, Epsom Surrey, UK), with the specific primers shown in supplemental Table 1. Fold inductions were calculated using the formula 2−[ΔΔCt], where ΔΔCt is the ΔCt (treatment) − ΔCt (control), ΔCt is Ct Has1–3, Gfat1, Gpi — Ct Arpo, and Ct is the cycle in which the detection threshold is crossed.

**Quantitation of Glicosaminoglycans with Dual Labeling**—100,000 cells were plated onto 12-well plates and cultured until nearly confluent. The medium was removed, and 0.45 ml of fresh medium containing 0 or 20 mM mannose was added to the wells. The radiolabels 200 μCi/ml [3H]glucosamine (PerkinElmer Life Sciences) and 1000 μCi/ml [35S]SO42− (Amersham Biosciences) were added in 50 μl of medium at 0, 3, 6, or 9 h after the medium change, and incubated a further 3 h before samples were harvested, as described (44). Briefly, the labeling medium, trypsin solution, and pelleted cells were analyzed by chromatographic separation of hyaluronan- and chondroitin sulfate-derived disaccharides following enzymatic digestion with hyaluronidase and chondroitinase and assessment of the specific activity of cellular [3H]hexosamines (45).

**UDP-hexose Analysis with Ion Pairing HPLC-MS/MS**—Cells seeded 2 days earlier and treated with various effectors for the indicated times were scraped off in an ice bath, pelleted at 4 °C,
Mannose and Hyaluronan Synthesis

and washed with cold PBS. Cold acetonitrile and water were sequentially added to the cell pellet to extract the sugar nucleotides and precipitate proteins. Alternatively, cells on plates were directly extracted with acetonitrile. Samples were centrifuged at 13000 \( \times g \) for 2 min at 4 °C, and the supernatant was evaporated using a vacuum centrifuge. Prior to analysis, samples were dissolved in 150 \( \mu l \) of MilliQ water containing 5 \( \mu M \) AppCp (Sigma M7510) as an internal standard.

The ion pairing HPLC-MS/MS measurements were carried out with a Finnigan Surveyor MS pump and autosampler and a Finnigan LTQ quadrupole ion trap mass spectrometer (Thermo Electron Corp., San Jose, CA). The separation was accomplished with a Gemini C18 reversed phase column (50 × 2.0 mm, 5 \( \mu M \)) (Phenomenex, Torrance, CA). Injection volume was 45 \( \mu l \). An eluent system of 20 mM dimethylhexylamine formate (Sigma) with pH adjusted to 6.8 (eluent A) and 80% methanol containing 2 mM dimethylhexylamine formate (eluent B) at a flow rate of 200 \( \mu l/min \) was used. The gradient was 2 min of eluent A, 0–100% of eluent B in 3 min, 100% eluent B for 4 min, and subsequent stabilization of the column with eluent A for 4 min. Electrospray and negative ion mode were used for the ionization of the molecules. Quantitation was based on multiple reaction monitoring of the characteristic fragment ions as follows: \( m/z \) 579 → 403 for UDP-GlcA (Sigma), \( m/z \) 606 → 385 for UDP-HexNac (Sigma), and \( m/z \) 504 → 406 for internal standard AppCp. The isomers UDP-GlcNAc and UDP-GalNac were not separated from each other in the chromatography and were quantified together.

Migration and Proliferation Assays—For migration analysis, 120,000 REK cells were seeded on 24-well plates coated with collagen IV (5 \( \mu g/ml \)) (BD Biosciences). After 24 h, an artificial wound was introduced with a pipette tip to the cell layer. Fresh medium with 10% fetal bovine serum and containing the factors to be examined were changed, and 6 h later the cultures were fixed with methanol. The cell-free area was measured immediately after the scraping and 6 h later using an Olympus CK2 inverted phase contrast microscope (Olympus Optical Co. Ltd., Tokyo, Japan), a Panasonic Ww CD 130-L video camera (Matsushita Electric Works, Tokyo, Japan), and NIH Image software. The newly covered wound area was calculated and converted to average migration distance from the wound edge.

For the proliferation analysis, 60,000 REK cells per well were seeded on 24-well plates, and after 4 h the appropriate concentrations of mannose were added to the cells. The media containing mannose were replaced each day. Cells were detached with trypsin-EDTA at 1–5 days following plating and counted with hemocytometer.

Protein Biosynthesis Assay—Nearly confluent cultures were changed into a methionine-free medium (Invitrogen) with 0.1 mCi/ml \( l-[35S] \) methionine (GE Healthcare). After 6-h incubations without additives (control), in the presence of 20 mM mannose or 2 mM GlCN, proteins were precipitated in 5% trichloroacetic acid, and incorporated radioactivity was measured by scintillation counting. Results were presented using the ratio of precipitated label to total radioactivity.

RESULTS

Mannose Inhibits Hyaluronan Synthesis in Keratinocyte Cultures—The common hexoses glucose, galactose, mannose, and fructose were added at 20 mM final concentrations to the basal culture medium of REKs for 24 h; the incubation media were collected, and secreted hyaluronan was analyzed with an ELSA (Fig. 1A). Although the other hexoses had relatively little effect (Fig. 1A), mannose caused a dose-dependent inhibition, reaching 45% with 15 mM concentration and 54% reduction at 20 mM (Fig. 1B). Cell-associated hyaluronan was also reduced by mannose, as shown by cytochemical staining of hyaluronan with a specific probe. After a 4-h treatment with 20 mM mannose, the cell-associated signal of hyaluronan was decreased (Fig. 1D), as compared with controls (Fig. 1C), and became even weaker after a 24-h treatment (Fig. 1E). Following a shift to mannose-free medium, the cultures showed complete restoration of hyaluronan synthesis (data not shown).

The Inhibition of Hyaluronan Synthesis Is Rapid—To obtain more information on the processes that lead to reduced hyaluronan production, we examined hyaluronan synthesis at different times following introduction of 20 mM mannose. Metabolic labeling in 3-h pulses over a 12-h period was used to monitor the rate of hyaluronan synthesis (Fig. 2A). The synthesis of hyaluronan was slightly stimulated by the change of fresh medium, as shown by the higher synthesis levels in control cultures during the 3–12-h time period (Fig. 2A), a finding consistent with earlier data on these cells (2). The inhibition occurred almost immediately after introduction of mannose, because the decrease in the 0–3-h labeling window was as large as during the later labeling periods (Fig. 2A). In contrast, the synthesis of chondroitin sulfates was not affected until in the 3–6-h labeling window and thereafter (Fig. 2B).

The Inhibition Does Not Involve Suppression of Has Expression—Alterations in hyaluronan synthesis in REK cells have often been associated with rapid changes in Has mRNA levels (2, 3, 27). Real time RT-PCR was therefore used to check possible contribution of lower Has expression in the inhibition of hyaluronan synthesis. Cells treated with 20 mM mannose and analyzed for the levels of Has1, Has2, and Has3 showed no decrease as compared with controls (data not shown). Fructose, galactose, and glucose did not have any marked effect on the expression of the three Has levels (data not shown). The early impact on hyaluronan synthesis without corresponding downregulation of Has expression indicates that mannose influences a step in HAS regulation beyond transcription.

Mannose Reduces UDP-HexNac Pool Size in Keratinocytes—A previously described hyaluronan synthesis inhibitor (4-MU), with an efficiency and selectivity closely resembling that of mannose (47), has been suggested to act through depletion of the cellular pool of UDP-GlcUA (35). Because the HAS enzymes use UDP-GlcUA and UDP-GlcNac as building blocks to construct the hyaluronan chain, the concentrations of the nucleotide sugars were measured as candidates for the inhibitory effect. Mannose reduced the UDP-HexNac pool in a dose-dependent way, with the maximum decrease of ~50%, whereas the UDP-GlcUA pool size was increased (Fig. 3A). Other hexoses like glucose, galactose, and fructose did not cause any
marked changes in the intracellular pools of UDP-HexNAc or UDP-GlcUA (data not shown). The reduction in UDP-HexNAc had occurred by 1.5 h following introduction of 20 mM man-

FIGURE 1. Effect of hexoses on hyaluronan synthesis in keratinocyte cultures. A, hyaluronan secreted into the growth media during a 24-h incubation in 20 mM galactose, glucose, fructose, or mannose was assayed by ELSA. The data represent means and ranges of three separate experiments. 8, hyaluronan secretion into growth medium of cells incubated for 24 h in the presence of 0–20 mM mannose. The data represent means ± S.E. of five separate experiments. Statistical significance between control and mannose-treated cultures is as follows: ***, p < 0.001, by the Tukey HSD test. C–E, cell-associated hyaluronan in nearly confluent REK cultures after 0-, 4-, and 24-h treatments of 20 mM mannose, visualized by staining with bHABC as described under “Experimental Procedures.”

Mannose and Hyaluronan Synthesis

MANNOSEx and HYALURONAN SYNTHESIS

4-MU Decreases Cellular UDP-GlcUA but Does Not Enhance the Inhibition of Hyaluronan Synthesis Caused by Mannose—Given the sensitivity of hyaluronan synthesis to the reduced concentration of UDP-HexNAc, as shown above, we compared the effect of mannose with that of 4-MU, which has been suggested to exert its inhibitory effect on hyaluronan synthesis by UDP-GlcUA depletion. Indeed, a dose-dependent reduction of UDP-GlcUA, down to 14% of control level, was found by 4-MU, whereas the level of UDP-GlcNAc was simultaneously reduced to 62% (Fig. 5A). The influence of 4-MU was also very rapid, reaching its greatest effect after 3 h (Fig. 5B).

Concentrations of 4-MU and mannose, which caused submaximal reductions in the UDP-hexose precursor pools, at 0.2 and 10 mM, respectively, were tested for their possible additive effects. Mannose and 4-MU together caused a depletion of both UDP-HexNAc and UDP-GlcUA, as expected (Fig. 5, C and D). Importantly, the synthesis of hyaluronan, measured after 6 (Fig. 5E) and 24 h (Fig. 5F), was only decreased to a level determined by the stronger of the two, with no additive effect (Fig. 5, E and F). This indicates that either one of the substrates can become rate-limiting and restrict the synthesis of hyaluronan.

Mannose Inhibits Keratinocyte Migration and Proliferation—Hyaluronan is involved in many keratinocyte functions such as migration, proliferation, and wound healing (5, 27, 46). To show the biological effects of the hyaluronan synthesis inhibition elicited by mannose, proliferation and migration rates were determined in REK cultures treated with mannose. Proliferation was determined in 0–20 mM mannose in 1–5 days by daily counting of the cells. Mannose treatment inhibited REK cell proliferation in a dose-dependent way (Fig. 6A). Cultures treated for 5 days with 10–20 mM mannose reduced the num-
Mannose and Hyaluronan Synthesis

FIGURE 2. Time course of hyaluronan synthesis after the addition of mannose in REK cultures. Nearly confluent cultures were incubated with [3H]glucosamine and [35S]sulfate for 3-h periods starting at different time points after addition of 20 mM mannose, and analyzed after enzymatic digestion into specific disaccharides as described under “Experimental Procedures.” A, combined total amounts of newly synthesized hyaluronan recovered in the medium, cell surface, and cell interior. The UDP-hexoses were quantified with ion pairing HPLC-MS/MS. The data represent means and ranges of two separate experiments. B, corresponding data of chondroitin/dermatan sulfates. The x axis bars show the period of synthesis examined, and the y axis bars indicate the range of two separate experiments.

FIGURE 3. Effect of mannose on UDP-HexNAc and UDP-GlcUA in keratinocyte cultures. A, nearly confluent keratinocyte cultures were incubated in 0–20 mM mannose for 3 h and analyzed for the content of UDP-HexNAc and UDP-GlcUA. B, changes in the content of the UDP sugars were analyzed in cultures treated with 20 mM mannose for 0–180 min. The UDP-hexoses were quantified with ion pairing HPLC-MS/MS. The data represent means and ranges of two separate experiments.

FIGURE 4. Glucosamine reversal of the mannose-induced decrease in UDP-HexNAc content and hyaluronan synthesis. REK cultures were incubated for 24 h in the presence of 20 mM mannose (Man), 2 mM glucosamine (GlcN), or both. A, content of UDP-HexNAc and UDP-GlcUA was assayed in two separate experiments with duplicate cultures in each. B, hyaluronan in the growth medium was determined in four experiments. The error bars show the range of the experiments in A and S.D. in B.

Inhibit REK cell proliferation more than either mannose or 4-MU alone, we treated REKs with mannose (10 mM) and 4-MU (0.2 mM) for 72 h with a daily change of culture media. 4-MU inhibited REK cell proliferation by about 35%, whereas mannose resulted in a smaller inhibition (9%), and 4-MU together with mannose caused about the same inhibition as 4-MU alone (Fig. 6B). 4-MU thus exerts a stronger inhibition as compared with mannose, and together they have no additive effect on cell proliferation.

To determine cell migration, confluent REK cultures were wounded by scraping with a pipette tip, and the wound areas covered after 6 h were determined for REK cells incubated with 20 mM concentrations of mannose or glucose. Although glucose did not affect REK cell migration (Fig. 6C), mannose reduced the migration rate by 31% (Fig. 6, C and D).

DISCUSSION

The present results show that deviations from the basal levels of cellular UDP-HexNAc are readily reflected in the synthesis of hyaluronan and that mannose inhibits hyaluronan synthesis because of its influence on the UDP-HexNAc pool size. We also verify the earlier suggestion that the cellular pool of UDP-GlcUA is dose-dependently depleted by 4-MU, a previously established inhibitor of hyaluronan synthesis. It was found that even relatively modest depletion of either UDP-HexNAc or UDP-GlcUA can inhibit hyaluronan synthesis, but reducing both at the same time does not increase the inhibition beyond that caused by the stronger of the two nucleotide sugars alone.

Mannose Inhibits Hyaluronan Synthesis through UDP-HexNAc Depletion—This is the first demonstration that mannose is a relatively potent inhibitor of hyaluronan synthesis. The inhibition was not because of its influences on the osmotic pressure of the culture medium or general increase in the energy supply of the cells, because closely related isomers of mannose, including glucose, galactose, and fructose, had no similar effect. Mannose was not toxic, and its effect on hyaluronan synthesis was also reversible.

A number of studies indicate that changes in hyaluronan synthesis are usually accompanied by corresponding alterations in the levels of mRNAs of one or more of the Has levels (2, 3, 25). Because Has mRNA levels were not reduced by mannose, and the full effect was obtained very rapidly, it is unlikely that the inhibition was because of changes in Has gene expression. Indeed, the finding that the inhibition of hyaluronan synthesis correlated with a markedly reduced cellular content of UDP-HexNAc fits well with the idea of shortage of UDP-GlcNAC, a substrate of HAS and a major building block of hyaluronan, being responsible for the inhibition. Although our mass spectrometric assay does not differentiate between UDP-GlcNAC
and UDP-GalNAc, both were likely reduced by mannose, because they exist in a free equilibrium, catalyzed by the enzyme UDP-galactose 4-epimerase (37). The magnitude and time scale of the reduction of the UDP-HexNAc-pool closely paralleled those of hyaluronan synthesis, findings that are also in line with the depletion of this sugar nucleotide as a mechanism in the inhibition of hyaluronan synthesis. The mechanism was confirmed by increasing the cellular UDP-HexNAc pool with exogenous glucosamine, which completely abrogated the inhibition by mannose.

Enzymatic Activity of HAS and Cellular Concentrations of UDP-GlcNAc and UDP-GlcUA—Analysis of recombinant mouse HAS1, HAS2, and HAS3 in membrane homogenates of COS-1 cells suggests that the $K_m$ values for UDP-GlcUA vary from 82 to 1011 μM and those of UDP-GlcNAc between 29 and 73 μM (47). Studies on recombinant Xenopus DG42 hyaluronan synthase resulted in similar $K_m$ values of 235 and 60 μM for UDP-GlcNAc and UDP-GlcUA, respectively (48). The expected affinity of UDP-GlcUA to HAS levels is thus 3–14 times higher than that of UDP-GlcNAc, suggesting that a correspondingly higher cellular concentration of UDP-GlcNAc is necessary for smooth synthesis of hyaluronan. The range of cellular UDP-GlcNAc/UDP-GlcUA ratios found in the present study (2–17 times more UDP-GlcNAc) and in several previous studies (36) is consistent with the idea that in the basal conditions neither substrate alone is consistently limiting the synthesis of hyaluronan.

Reducing the expression of UGDH, the enzyme that produces UDP-GlcUA, by transfection with short interfering RNA inhibits hyaluronan synthesis in smooth muscle cells (34). Accordingly, transfection and overexpression of (sense) UGDH cDNA slightly increase hyaluronan synthesis (34). The present data indicate that the same rule applies to UDP-GlcNAc; reducing its content inhibits hyaluronan synthesis, whereas a significant increase of hyaluronan synthesis is obtained by expanding the pool of UDP-GlcNAc. The need of equimolar amounts of the two substrates also means that increasing either one alone has a limited stimulatory effect on hyaluronan synthesis.

**FIGURE 5.** The effects of 4-MU and 4-MU combined with mannose on UDP-hexoses and hyaluronan synthesis. A, concentration dependence of the 4-MU influence on the UDP-hexoses was studied in REK cells incubated in 0.2–1.0 mM 4-MU for 9 h. B, influence of 0.5 mM 4-MU on the UDP-hexoses was analyzed 3–12 h after introduction of the inhibitor. The combined effects of 10 mM mannose and 0.2 mM 4-MU during 6 h of treatment on UDP-GlcUA (C) and UDP-HexNAc (D) were studied with ion pairing HPLC-MS/MS. The bars in A–D show the range of two separate experiments. E and F, cells were incubated in 10 mM mannose (Man) and 0.2 mM 4-MU for 6 (E) and 24 h (F), and hyaluronan concentration in the medium was analyzed with ELISA. The data represent means ± S.E. of five separate experiments. *, $p < 0.05$ as compared with controls (Tukey HSD test).

**FIGURE 6.** Keratinocyte proliferation and migration in the presence of mannose. A, for proliferation assays, cells were incubated in 0–20 mM mannose for 1–5 days and counted in a hemocytometer. A new culture medium, including mannose, was changed every day. Means ± S.E. of three separate experiments are shown. B, combined effects of 10 mM mannose and 0.2 mM 4-MU on cell numbers were studied after 3 days of treatment. C, REK migration in the presence of 20 mM mannose and glucose was compared in a scratch wound model as described under “Experimental Procedures.” Means ± S.E. in three separate experiments are shown. D, for statistical analysis, a separate set of five experiments was done on the mannose-induced inhibition on migration. Means ± S.E. are shown. ***, $p < 0.001$, paired samples t test. **,
Mannose and Hyaluronan Synthesis

Specificity of Hyaluronan Synthesis Inhibition to UDP-HexNAc Depletion—The present findings indicate that the response of hyaluronan synthesis to the introduction of mannose is rapid and strong. On the other hand, the synthesis of chondroitin sulfates, glycosaminoglycans also dependent on UDP-HexNAc and UDP-GlcUA, first proceeded at an unchanged rate and was generally less affected by the depletion of UDP-HexNAc. This finding may be due to the fact that hyaluronan synthesis utilizes UDP sugars that reside in the cytosol, whereas the synthesis of chondroitin sulfates takes place in the Golgi apparatus. UDP sugars are pumped from cytosol into Golgi apparatus by transporters that have some specificity to UDP sugars. UDP sugars in the Golgi apparatus synthesize hyaluronan and chondroitin sulfates, glycosaminoglycans also dependent on UDP-GlcUA and UDP-HexNAc. This finding may be due to the fact that hyaluronan synthesis utilizes UDP sugars that reside in the cytosol, whereas UDP-HexNAc and UDP-GlcUA, first proceeded at an unchanged rate and was generally less affected by the depletion of UDP-HexNAc (34, 35, 46). This specific sensitivity of hyaluronan synthesis to the reduction of UDP-GlcUA can also be explained by a lower concentration of this UDP sugar in the cytosol than in Golgi apparatus (34).

Possible Targets of the Mannose-induced UDP-HexNAc Reduction—It is reasonable to assume that there is a specific feature in the structure of mannose or its metabolite that is recognized by the cell, because a similar response was not obtained with other monosaccharides. The most likely targets of mannose are enzymes responsible for the synthesis and metabolism of UDP-GlcNAc.

The first and most important step in the synthesis of UDP-HexNAc is catalyzed by the isoenzymes GFAT1 and GFAT2, resulting in the formation of GlcN-6P. Acetylation of the amino group, shift of the phosphate to carbon 1 (GlcN-1P), and reaction with UTP then produces UDP-GlcNAc. The reactions are generally reversible, except for that mediated by GFAT. Because exogenous GlcN joins this biosynthetic pathway at the step of GlcN-6P, bypassing GFAT, and completely abrogates the effect of mannose on UDP-GlcNAc content, it is unlikely that the target of mannose, if in the biosynthesis of hexosamines, resides between GlcN-6P and UDP-GlcNAc. That leaves GFAT as a possible target of mannose. Because the mRNA level of Gfat1 was not affected, and Gfat2 was not expressed in REKs, at least transcriptional Gfat regulation as a target of mannose was excluded.

There is another ubiquitous enzyme, glucosamine-6-phosphate deaminase (GPI), the suggested function of which is to return excess hexosamines back to fructose 6-phosphate and release the amino group as NH₃ (51). This reaction can run to both directions depending on the substrate concentrations, resulting in either net synthesis or degradation of GlcN-6P. Although transcriptional regulation of GPI was ruled out, activation of the GlcN-6P deaminase reaction in the catabolic direction could still account for the reduced UDP-GlcNAc content. Interestingly, mannose 6-phosphate reportedly regulates this enzyme (40), offering a good explanation for the observed influences of mannose.

Importance of the Ability of Mannose to Inhibit Hyaluronan Synthesis—Hyaluronan synthesis has been associated with cell proliferation and migration in many tissues and in many types of cultured cells (52), including keratinocytes (2, 53). Accordingly, in this study the inhibition of hyaluronan synthesis by mannose is associated with the reduced rate of cell migration and proliferation. A similar inhibition of hyaluronan synthesis was noted in a number of other cell lines, including fibroblasts, melanoma cells, and keratinocytes, indicating that the target of mannose is ubiquitous and the response similar irrespective of the cell type. At the moment, we have no data on its effects in vivo, but this will be explored in the future, because there are a number of conditions in which hyaluronan accumulation is considered deleterious, and reducing its content may be of clinical benefit. For instance, increased hyaluronan expression is associated with acute or chronic inflammatory conditions (54), and hyaluronan may facilitate anchorage-independent growth, malignant invasion, and metastasis (13). As an example of the feasibility of this idea, experiments have already been published in which reducing the cellular content of UDP-GlcUA inhibits cancer growth and metastasis (55). However, because of the relatively high concentrations needed, it is unlikely that systemic administration is feasible, although feeding of mannose is clinically effective and well tolerated in the treatment of patients with carbohydrate-deficient glycoprotein syndrome (56).

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