Hyperglycemia Diverts Dividing Osteoblastic Precursor Cells to an Adipogenic Pathway and Induces Synthesis of a Hyaluronan Matrix That Is Adhesive for Monocytes

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Background: Adipocytes accumulate in diabetic bone marrow.

Results: Bone marrow stromal cells that divide in hyperglycemia divert from the osteoblast lineage to pathological adipogenesis and produce an extensive monocyte-adhesive hyaluronan matrix with consequent demineralization of trabecular bone.

Conclusion: This mechanism diminishes the stromal cell population with an accumulation of metabolically stressed adipocytes.

Significance: This provides new insights into diabetic osteopenia.

Osteopenia and an increased risk of hip fracture are associated with type 1 diabetes in both humans and experimental animals (1-8). Previous studies have also shown decreased bone formation accompanied by reduced mineral content in experimental models of type 1 diabetes mellitus as well as in spontaneous diabetes (6-8). Several possibilities have been proposed, including insulinopenia, bone microangiopathy, impaired regulation of mineral metabolism, alterations in local factors that regulate bone remodeling, and even an intrinsic disorder associated with type 1 diabetes mellitus (9). However, impaired bone formation is considered one of most important factors in the development of diabetic osteopenia.

The underlying mechanisms for bone loss associated with diabetes are not completely understood. In diabetic patients, decreased osteoblast function is thought to be associated with a defect of osteocyte maturation on the basis of normal serum levels of procollagen peptide, an early osteoblast marker, and decreased serum levels of osteocalcin, a late-stage marker of osteoblast maturation (10, 11). The decrease in serum osteocalcin is even more pronounced in diabetic rat models (5, 12-14). These data suggest that a decrease in osteoblast number, function, and/or maturation may be a contributor(s) to bone loss in diabetes (10). Previous studies have also shown that significant trabecular bone loss and increased bone marrow adiposity occur in both streptozotocin-induced and nonobese diabetic mouse models (10, 15). Because both osteoblasts and adipocytes are derived from the same mesenchymal stromal cells located in the bone marrow (16, 17), these studies suggest that diabetic hyperglycemia may promote the selection of the adipocyte over the osteoblast lineage during stromal cell differentiation (10, 15). The decrease in mature osteoblasts and increase in adipocytes in diabetic bone marrow may then contribute to the loss of trabecular bone by decreasing the number of osteoblasts available for proper bone remodeling.

Hyaluronan is a linear glycosaminoglycan composed of repeating disaccharide units of N-acetylgalactosamine and glucuronic acid. It is normally synthesized at the inner side of the plasma membrane by hyaluronan synthases (HASs) that alternately add cytosolic UDP-GlcUA and UDP-GlcNAc to the reducing end of the growing chain, which is extruded directly into the extracellular space and can reach sizes greater than 10 MDa (18-21). There are three homologous hyaluronan syn-
these proteins (HAS1, 2, and 3), each with several transmembrane domains and a central cytoplasmic region that can be phosphorylated (23). They are normally transported in an inactive form to the plasma membrane before activation to the active form to the plasma membrane before activation to synthesize and extrude the hyaluronan chain.

Autophagy is a cell response that involves endoplasmic reticulum stress and is an attempt by the cell to dispose of unfolded proteins by transport to aggresomes that contain proteasomes (24). Our previous studies showed that rat glomerular mesangial cells stimulated to divide in hyperglycemic medium (21, 25, 26) initiate intracellular synthesis of hyaluronan during the cell cycle; up-regulate cyclin D3 and C/EBPα concurrently with extrusion of the matrix; and form aggresomes and autophagosomes that coalesce for hyaluronan, cyclin D3, C/EBPα and microtubule-associated light chain 3 (LC3), a marker for autophagy.

Our results in this study indicate that rat bone marrow stromal cells (RMSCs) dividing in hyperglycemic medium undergo this autophagy mechanism and divert from an osteogenic to a chondrogenic response that also correlates with extrusion of the hyaluronan matrix after completing the cell cycle; induce an autophagic response that also correlates with extrusion of the matrix; and form aggresomes and autophagosomes that coalesce for hyaluronan, cyclin D3, C/EBPα and microtubule-associated light chain 3 (LC3), a marker for autophagy.

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. Antibody against KDEL (the sequence of Lys-Asp-Glu-Leu) was from Affinity Bioreagents (Golden, CO) (27). Antibodies against C/EBPα, PPARγ, HAS2, MAC, CD44, and microtubule-associated protein 1 light chain 3 (LC3) were from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-RUNX2 antibody was from Abcam (Cambridge, MA).

Establishment of RMSC Cultures and Induction of Diabetes in Rats—Hyperglycemic diabetes was induced in ~175-g male Sprague-Dawley rats using tail vein injections of 55 mg/kg streptozotocin (28, 29). All animals were fed a 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 (30). After 4 weeks, the tibiae were collected from both control and diabetic rats and fixed in 75% cold ethanol at 4 °C overnight for subsequent micro-CT scanning and for demineralization and sectioning for histological analyses (Histochemistry Core Facility, Department of Biomedical Engineering, Cleveland Clinic).

RMSC cultures were established from isolated 1-month-old rat tibiae as described previously (31). The cells were expanded in mesenchymal stem cell expansion medium (5.6 mM glucose) (Millipore) according to the instructions of the manufacturer. For monolayer cultures, RMSCs were used between passages 4 and 10. To render cells quiescent, cultures at ~40% confluence were washed with DMEM and placed in fresh expansion medium containing 0.4% FBS for 48 h (yielding about 80% confluent cultures) (25, 30). The cultures were then stimulated to divide with 10% FBS in mesenchymal stem cell osteogenesis medium optimized for rats (Millipore) with 0.1 μM dexamethasone, 0.2 mM ascorbic acid 2-phosphate, and 10 mM glycerol 2-phosphate in the presence of 5.6 mM or 25.6 mM glucose or 5.6 mM glucose plus 20.0 mM mannitol. In some experiments, RMSC cultures were treated with 1 μg/ml heparin (high + heparin), with 0.25 mM 4-methylumbelliferyl-β-D-xyloside (4-MU-xyI), or with a PKC inhibitor, 100 nM bisindolylmaleimide I, in the osteogenesis medium containing 25.6 mM glucose.

Micro-CT Analysis—Isolated rat tibia in 75% ethanol were placed on the scanning bed of a GE eXplore Locus Micro CT (GE Healthcare), and 360 x-ray projections were collected in 1° increments (80 kV, 500 μA, 26 min of total scan time). Projection images were preprocessed and reconstructed into three-dimensional volumes (10243 voxels, 20-μm resolution) on a four-computer reconstruction cluster using a modified tent Felkamp-Davis-Kress cone beam algorithm (GE Reconstruction Software). Three-dimensional data were processed and rendered (isosurface/maximum intensity projections) using MicroView (GE Healthcare).

Tibial segmentation, volume enhancement, and delineation of regions of interest (ROIs) were done in MicroView. For each sample, a plane perpendicular to the z axis/tibial shaft was generated and placed along the growth plate 1 mm below the cartilage. A second, parallel plane was defined 3 mm below the first, and the entire volume was clipped to this ROI. Image stacks from each ROI were exported for quantitative analysis.

For the extraction of three-dimensional trabecular structural indices (customized algorithms in MatLab), a semiautomated routine was applied to each ROI to generate a mask of the cancellous bone space. This step involves a slice-by-slice application of a global threshold to segment cortical bone, morphological filters to remove objects within the marrow space, and a connected components labeling algorithm to validate the presence of contiguous cortical bone. When a cortical bone mask is generated, its complement is used to define the cancellous bone space. This resultant mask is multiplied by the original ROI to extract trabecular bone. Bone volume fraction (BV/TV, total bone voxels divided by total trabecular volume mask voxels) and mineral density were calculated for each ROI as described in previous studies (32, 33).

Immunohistochemistry—Decalcified paraffin-embedded tibia sections or methanol-fixed RMSC cultures on cover slips were stained for hyaluronan with a hyaluronan-binding protein (Seikagaku America); for cyclin D3, C/EBPα, PPARγ, CD44, MAC, and LC3 with antibodies; and for nuclei with 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 microscope 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 Systems (San Jose, CA).

In some experiments, RMSC cultures were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and then stained with Nile Red as described previously (34). In other experiments, RMSC cultures were stained with Alizarin Red S for biomineralization (35, 36) and with Oil Red O for lipid accumulation (37).

**Assay for Monocyte Adhesion**—RMSCs in 6-well plates were treated up to 5 days with 10% 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 passaged at a 1:5 ratio (2 \times 10^5 cells/ml) every 48 h (38). Assays for monocyte adhesion were done at 4 °C as described previously (30, 38). After washing, the cell cultures were imaged by microscopy with a Polaroid digital camera (30), and the numbers of monocytes per culture area were counted using Image-Pro software. Each culture was equally divided into four regions, and a culture area for imaging was randomly picked in each region. Streptomyces hyaluronidase (1 turbidity reducing units/ml at 37 °C for 15 min) treatment of RMSCs before monocyte incubation was used to determine the extent of the hyaluronan-mediated adhesion.

**FACE Analyses**—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. Glycosaminoglycans were recovered by 75% ethanol precipitation at −20 °C overnight and centrifugation. The pellets were dissolved in 0.1 M ammonium acetate (pH 7.0) and incubated with streptococcal hyaluronidase (50 milliunit/ml) and chondroitinase ABC (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 sample digests were dried by centrifugal evaporation in microtubes and then subjected to reductive amination with 2-aminoacridone as described previously (39, 40). At the end of the incubation, the samples were mixed with glycerol to 20%, and 5-μl aliquots were electrophoresed 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 on 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 intensity of signal bands and then normalized with DNA contents in the samples.

**Western Blot Analysis**—For Western blot analysis, RMSCs were lysed with the Laemmli sample buffer. Total cell extracts were separated by a 10-well polyacrylamide gel (catalog no. NP03Z21BOX, Invitrogen) and blotted to nitrocellulose (catalog no. 926-31090, Li-Cor, Lincoln, NE). The membranes were probed as described previously (27) with the indicated antibodies and corresponding secondary antibodies. The blots were washed and imaged on an Odyssey infrared imaging system (Li-Cor) (27).

**RESULTS**

**Osteopenia in 4-week-old Diabetic Rat Tibia**—Micro-CT sagittal slices (A and B) and three-dimensional, segmented trabecular structure (C and D) show extensive demineralization in the diabetic cancellous bone (boxes) compared with the control. Autofluorescence of demineralized sections in the same trabecular bone region shows little difference (E and F).

**FIGURE 1. Demineralization in trabecular bone in 4-week-old diabetic rat tibia.** Micro-CT sagittal slices (A and B) and three-dimensional, segmented trabecular structure (C and D) show extensive demineralization in the diabetic cancellous bone (boxes) compared with the control. Autofluorescence of demineralized sections in the same trabecular bone region shows little difference (E and F).
divided by total trabecular volume mask voxels) and a $9 \pm 1.6\%$ ($n = 3$) decrease in bone mineral density, consistent with previous studies (10, 15). Demineralized sections from the same region of interest were evaluated by H&E staining and autofluorescence. The autofluorescence shows patterns characteristic of the underlying collagen matrix in trabecular bone regions of both the diabetic and control (Fig. 1, E and F). The H&E decalcified stained sections also show structures characteristic of bone trabecula in the diabetic bone (Fig. 2, arrows). These results suggest that the loss of mineral in this region is not characteristic of a normal osteoclastic response (41), which would also remove the underlying collagen matrix (42). Higher magnifications of the sections (Fig. 2, C and D) show that the diabetic trabecular bone region contains numerous large spherical cells (asterisks) that are not present in the same region of the control trabecular bone (10, 15). Fig. 2F shows an enlarged view of the cluster of these cells. Figs. 3 and 4 provide evidence that these large spherical cells have staining characteristics of adipocytes.

Hyaluronan Matrix and Autophagic Adipocytes in the Trabecular Bone Region of a 4-week-old Diabetic Rat Femur—Demineralized sections of trabecular bone were stained for hyaluronan (green) and either cyclin D3 (red) (Fig. 3, A and B) or C/EBPα (Fig. 3, C and D) (red). An extensive hyaluronan matrix is present in the diabetic bone marrow and present at much lower levels in the control. Several large, round cells that stain heavily for cyclin D3 and C/EBPα are embedded in the hyaluronan matrix of the diabetic bone, which also contains numerous small mononuclear cells (DAPI-stained nuclei) characteristic of the hematopoietic cells (monocytes/macrophages). Fig. 3, E and F, shows sections stained for cyclin D3 (red) and microtubule-associated protein 1, light chain 3 (LC3) (green), a marker for autophagy. Colocalization of these proteins (yellow, Fig. 3F) show structures (asterisks) in the large cells that appear characteristic for coalescence of autophagosomes, as shown in our previous study with mesangial cells that were stimulated to divide in hyperglycemic medium (21, 25, 26). Neither the extensive hyaluronan matrix nor the large cells are present in the control bone sections. The results suggest that the large cells are adipocytes that have undergone autophagy and that they are likely involved in the production of the hyaluronan matrix.
CD44- and MAC-stained Cells in the Hyaluronan Matrix

Fig. 4 shows sections of control (A, merged) and diabetic (B–D) of bone marrow stained for hyaluronan (green) and CD44 (red), a cell surface hyaluronan receptor. At 4 weeks, the diabetic bone marrow contains an extensive hyaluronan matrix (Fig. 4B) with large numbers of mononuclear cells (Fig. 4C) that are embedded in the hyaluronan matrix with their cell surface CD44 molecules closely associated with the hyaluronan (Fig. 4D). Sections from control bone marrow show minimal staining (Fig. 4A). Fig. 4 also shows sections of control (Fig. 4E, merged) and diabetic (Fig. 4, F–H) bone marrow stained for hyaluronan (Fig. 4F) and for cells stained for MAC (Fig. 4G), a marker for macrophages, that are embedded in the hyaluronan matrix (Fig. 4H). A number of the large adipocytes (asterisks, Fig. 4H) are also present in the hyaluronan matrix. These results are similar to other inflammatory responses in which metabolically stressed cells produce a hyaluronan matrix that recruits monocytes/macrophages (21, 25, 26, 38, 43, 44), and they suggest that the sustained hyperglycemia has initiated a chronic inflammatory response that produces an abnormal hyaluronan matrix that recruits macrophages, which may be responsible for the loss of mineral (41).

Rat Bone Marrow Stromal Cell Cultures in Normal and Hyperglycemic Osteogenic Medium (Days 21–31)

On the basis of the results in the sections from the 4-week-old diabetic bone, our hypothesis is that bone marrow stromal cells that divide in hyperglycemic medium divert to an autophagic adipogenic phenotype and produce the hyaluronan matrix that recruits monocytes/macrophages. To test this, we cultured rat bone marrow stromal cells in standard osteogenic medium that is used to prepare osteoblasts (5.6 mM glucose) and in the same medium with hyperglycemic glucose (25.6 mM) or with normal glucose plus 20 mM mannitol as an osmotic control. Fig. 5 shows cultures stained for mineralization (Alizarin Red) and for lipids (Oil Red O). The culture in normal osteogenic medium showed the characteristic increase in mineral between days 21–31, with only light staining for lipids during this time, indicating that the osteogenic medium with normal glucose (5.6 mM) promoted osteogenesis. In contrast, the osteogenic medium with hyperglycemic glucose showed an extensive accumulation of lipids during the same time period and much less mineralization. The mannitol osmotic control showed reduced mineral deposition and no lipid accumulation. The results with mannitol suggest that either the osmotic conditions or some response to mannitol prevents the mineralization but also indicate that the cells did not stimulate lipid synthesis.

Rat Bone Marrow Stromal Cell Cultures in Normal and Hyperglycemic Osteogenic Medium (Day 5)—RMSCs were cultured for 5 days in osteogenic medium with normal glucose,
Hyperglycemia Induces Hyaluronan Synthesis

Figure 6. RMSCs increase synthesis of lipids, hyaluronan, and cyclin D3 when normal (5.6 mM glucose) osteogenic medium is increased to hyperglycemic (25.6 mM). Rat marrow stromal cells cultured for 5 days in osteogenic medium with normal (5.6 mM) glucose (A, D, and G), hyperglycemic (25.6 mM) glucose (B, E, and H), or normal glucose plus 20 mM mannitol (C, F, and I) were stained with Nile Red (A–C) or permeabilized and stained for cyclin D3 (red) and either hyaluronan (green, D–F) or LC3 (green, G–I).

hyperglycemic glucose, or normal glucose plus mannitol as described under "Experimental Procedures." Fig. 6 shows 5-day cultures stained with Nile Red (Fig. 6, A–C), which stains neutral lipids (yellow-green) and phospholipids (orange-red). The extensive accumulation of both classes of lipids in the hyperglycemic culture (Fig. 6B), but not in the normal glucose and mannitol control cultures (Fig. 6, A and C), is apparent.

Fig. 6 also shows cultures with permeabilized cells stained for hyaluronan (green) and cyclin D3 (red) (Fig. 6, D–F). The hyperglycemic culture (Fig. 6E) contains an extensive hyaluronan matrix, and a majority of the embedded cells contain aggresomes stained for cyclin D3. These responses are nearly absent for the normal glucose culture (Fig. 6D) and the mannitol culture (Fig. 6F). The autophagic response is further demonstrated by the colocalization of LC3 and cyclin D3 in the hyperglycemic culture (Fig. 6H) and its near absence in the other two treatments (Fig. 6, G and I).

Fig. 7 shows adhesion of U937 monocytes to RMSC cultures at 5 days after the indicated treatments, and quantification of the results is shown in the bar graphs (three cultures for each treatment and for cultures treated with Streptococcus hyaluronidase before adding the U937 monocytes). Significantly more monocytes bind to the cultures in hyperglycemic medium (high) than for cultures in medium with normal glucose (low). Hyperglycemic medium with 4-MU-xyl, which diverts cytosolic UDP sugar substrates into the Golgi to synthesize extensive amounts of chondroitin sulfate (45), showed no increase in monocyte adhesion (high + MU-xyl). Similarly, hyperglycemic medium with the PKC inhibitor bisindolylmaleimide I, which inhibits the activation of the HAS and the autophagy during cell division in kidney mesangial cells (30), showed no increase in monocyte adhesion. In contrast, hyperglycemic medium with heparin, which has been shown to prevent nephropathy and proteinuria in the streptozotocin diabetic rat (46, 47), significantly increased monocyte adhesion above the cultures treated with hyperglycemia alone, as shown in the bar graphs (Fig. 7). This result is consistent with the results with mesangial cells.4

The increases in monocyte adhesion in both of these hyperglycemic treatments were prevented when the cultures were first treated with the hyaluronidase (black bars compared with white bars, Fig 7).

Fig. 8 shows the results for hyaluronan analyses of the cell layers of 5-day RMSC cultures treated with these protocols, with an example of a FACE analysis for one set of cultures. As expected from the monocyte adhesion results (Fig. 7), the hyaluronan contents for the hyperglycemic cultures increased significantly compared with the cultures in normal glucose medium (high compared with low), and the presence of heparin significantly further increased the hyaluronan contents. The hyaluronan contents for both the 4-MU-xyl- and PKC inhibitor-treated hyperglycemic cultures were not significantly different from the cultures in normal glucose. These results show that the hyaluronan matrix produced by the RMSCs in the hyperglycemic medium has structural configurations that monocytes recognize and adhere to, indicative of a pathological monocyte-adhesive hyaluronan matrix (21).

Interestingly, 3T3-L1 cells, which are considered a standard model for studying adipogenesis, are routinely stimulated to divide in hyperglycemic medium to induce adipogenesis. A previous study (49) showed that the culture medium after cell division contained a highly viscous hyaluronan matrix. Fig. 9 (reproduced with permission from our FEBS Journal review (21)) shows that the 3T3-L1 cells in this standard protocol up-regulate cyclin D3, undergo autophagy, and form a monocyte-adhesive hyaluronan matrix essentially identical to the responses of the stromal cells in Figs. 6 and 7.

Fig. 10 shows an example of the Western blot analyses for the indicated proteins and bar graphs for analyses from three cultures for each of the indicated treatments normalized to the β-actin bands. C/EBPα and PPARγ, markers for adipocytes (50–53), were up-regulated in the hyperglycemic cultures but not in the hyperglycemic cultures treated with the 4-MU-xyl or the PKC inhibitor. Similarly, GRP94, a marker for ER stress, and cyclin D3 were also significantly up-regulated only in the hyperglycemic medium alone. RUNX (Runt-related transcription factor), which would be critical for osteogenesis at a later stage, was not altered significantly in any of the hyperglycemic medium treatments. Interestingly, hyaluronan synthase 2 (HAS2) was increased significantly in the hyperglycemic medium, as shown previously for mesangial cells (54), but not in the hyperglycemic medium with heparin. This indicates that

4 A. Wang, J. Ren, C. P. Wang, and V. C. Hascall (2014) 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. J. Biol. Chem. 289, 9418–9429.
the significant increase in hyaluronan synthesized by the hyperglycemic RMSCs in the presence of heparin is not due to an increased HAS2 translational response. It is possible that the increase in HAS2 in the hyperglycemic medium alone, although inducing less hyaluronan synthesis, may relate to the disruption in the intracellular trafficking of the HAS2 because of its activation in the intracellular compartments. Further experiments are needed to address this dichotomy.

DISCUSSION

Previous studies of diabetic bone marrow have suggested that the interplay between various events at both the cellular and molecular levels result in the progression of diabetic bone loss (55). These events include a decrease in osteoblast differentiation/maturation (1, 55, 56); an increase in adipocyte differentiation (10, 15, 55); an increase in monocyte/macrophage activation (57); an increase in bone inflammation (55, 57); and a decrease or no change in osteoclast number, erosion depth, and erosion surface (12, 13, 55, 58). However, the mechanisms underlying diabetic bone mineral loss are still unclear. Our study provides compelling evidence for a causal link between the response of dividing bone marrow stromal cells to hyperglycemia and their diversion to adipogenic responses instead of osteogenic pathways with the synthesis of an extensive monocyte-adhesive hyaluronan matrix in diabetic cancellous bone marrow that recruits monocytes/macrophages.

Mature osteoclasts are differentiated from the monocyte and macrophage lineage. In hyperglycemia, however, monocytes/macrophages can initiate inflammatory responses instead of the formation of mature osteoclasts. In a chronic inflammation, macrophage activation can lead to localized low pH, which can promote local bone demineralization (41, 42, 59, 60). The excessive numbers of CD44- and MAC-positive mononuclear cells in the abnormal hyaluronan matrix in the diabetic bone marrow are likely to be monocytes/macrophages that have...
entered this matrix. Thus, the increased abnormal hyaluronan matrix in the trabecular bone region and bone marrow in response to hyperglycemia could mediate monocyte/macrophage adhesion and promote an inflammatory phenotype. This could then lead to lower local pH and demineralization of adjacent trabecular bone (41), a possible cause of diabetic bone mineral loss, without proportional removal of the underlying collagen matrix.

Both adipocytes and osteoblasts are differentiated from mesenchymal stromal cells located in the bone marrow (16, 17, 51). The mechanism(s) underlying the conversion of multipotent stem cells into the adipocyte lineage are still unknown. Previous studies have suggested that the hyperglycemia could be one of the factors involved in this process (10, 15). Our data show that hyperglycemia in osteogenic medium induces adipogenic responses of RMSCs instead of the normal osteogenic responses. This strongly suggests that there is a diversion or interconversion of osteogenic commitment to an adipogenic pathway at the preosteoblast stage. Further, it is likely that both daughter cells divert to adipogenesis after division, which would decrease the number of stromal cells available for osteoblast conversion.

The early increases in expression of cyclin D3, C/EBPα, and PPARγ in the hyperglycemic RMSC cultures (within 5 days) clearly indicate this adipogenic conversion. Previous studies have shown that cyclin D3 is increased during adipogenic differentiation of 3T3-L1 cells (50), which undergo the same responses because of division in hyperglycemic medium (Fig. 9) (21). Cyclin D3 phosphorylates C/EBPα and interacts with PPARγ as a ligand-dependent coactivator. Pertinent to our study, previous studies have shown that overexpression of cyclin D3 in 3T3-L1 cells stimulates adipogenesis, whereas knockdown studies inhibited adipogenesis (50). During adipogenesis, C/EBPα and PPARγ act synergistically to activate the transcription of genes that produce the adipocyte phenotype (51–53). Overexpression of PPARγ in osteoblasts suppresses the mature osteoblast phenotype and induces genes associated with an adipocyte phenotype (61, 62). Thus, taken together, our studies demonstrate that hyperglycemia promotes the selection of adipogenesis over osteoblastogenesis of bone marrow stromal cells and that this selection process may contribute to dia-
betic bone loss in conditions such as age-related and disuse-associated osteoporosis (10, 63–68).

The formation of an extensive hyaluronan matrix with embedded macrophages has been demonstrated in the adipose tissue of a diabetic mouse model (69). Our studies with rat diabetic bone tissue demonstrate that adipogenesis and hyaluronan matrix with embedded macrophages also occurs in the diabetic trabecular bone marrow, most likely through diversion of dividing stromal cells to adipogenesis. This, thus, could have a major impact on impairing fracture healing, a known problem for diabetics (70–72), by diverting precursor cells necessary to provide osteoblasts for bone repair to the adipogenic pathway and/or by depleting the reservoir of stromal cells during chronic responses over time to hyperglycemia. Therefore, variations in synthesis of this abnormal hyaluronan matrix likely contribute to many diabetic pathologies.

Chronic hyperglycemia in diabetes causes excessive amounts of intracellular glucose and its metabolites, which can activate intracellular metabolic pathways that lead to diabetic complications (22). The elevated cytosolic UDP-GlcNAc concentration has been suggested as one of the major mediators of diabetic pathology (48), but its roles in adipogenesis induced by hyperglycemic glucose is still not clear. Our results identify two possible ways to modulate the intracellular UDP-GlcNAc: treatment with heparin and with 4-MU-xyl. Heparin blocks the activation of the HAS enzymes in cytosolic membranes during cell division, possibly by interfering with the PKC signaling pathway involved. After cell division, heparin initiates an as yet unknown signaling pathway that stimulates the formation of an extensive monocyte-adhesive HA matrix using UDP-GlcNAc as one of substrates, which would diminish the concentrations of the cytosolic UDP sugars during continued glucose stress. 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 (45). Our data show that both heparin and 4-MU-xyl inhibit hyperglycemia-induced adipogenic responses as well as the expressions of cyclin D3, C/EBPα, and PPARγ without affecting RUNX2 expression, a marker for osteogenesis. This strongly suggests that cytosolic UDP-GlcNAc and, possibly, UDP-GlcUA have critical roles in mediating the diversion or interconversion of osteogenic commitment to an adipogenic pathway at the preosteoblast stage. Also, 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 diabetic pathology.

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