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Diabetes Interferes with the Bone Formation by Affecting the Expression of Transcription Factors that Regulate Osteoblast Differentiation

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Type 1 diabetes in humans has as one of its complications inadequate bone formation, resulting in osteopenia and delayed fracture healing. To investigate the mechanisms by which diabetes affects bone formation, experiments were performed in a marrow ablation model. Mice were made diabetic by multiple low-dose streptozotocin treatment, and controls were treated with vehicle alone. Killing occurred 0, 2, 4, 6, 10, and 16 d following marrow ablation. Histologic analysis demonstrated that the amount of immature mesenchymal tissue was equivalent in both the experimental and control groups on d 4. On d 6 a burst of bone formation occurred in the control group that was significantly reduced in the diabetic group. This deficit was evident at the molecular level as shown by diminished expression of osteocalcin, collagen types I. When transcription factors were examined, core-binding factor α1 (Cbfa1)/runt domain factor-2 (Runx-2) and human homolog of the *drosophila* distal-less gene (Dlx5) expression were substantially reduced in the diabetic, compared with control, groups on d 4 and 6. C-fos but not c-jun expression was also suppressed in the diabetic group but not closely linked to bone formation. Insulin treatment substantially reversed the effect of diabetes on the expression of bone matrix osteocalcin and collagen type I and transcription factors Cbfa1/Runx2 and Dlx5. These results indicate that diabetic animals produce sufficient amounts of immature mesenchymal tissue but fail to adequately express genes that regulate osteoblast differentiation, Cbfa1/Runx-2 and Dlx5, which in turn, leads to decreased bone formation. (*Endocrinology* 144: 346–352, 2003)

IN HUMANS, TYPE 1 DIABETES is associated with a decrease in skeletal mass and delayed healing of fractures (1–3). Diabetic patients exhibit reduced bone mineral density as measured by dual x-ray absorptiometry at the lumbar spine and proximal femur (4). In a 12-yr prospective study, bone formation was shown to be reduced in diabetic humans, suggesting that they have diminished osteoblast activity, compared with normal individuals (5).

Insight into the mechanisms of diabetes-associated delays in fracture healing comes largely from studies using animal models. Several of models of type 1 diabetes have shown reduced bone turnover impaired fracture repair as measured biomechanically by torque to failure and deficits in mineralization (6–8). In streptozotocin-induced diabetic rats, abnormal bone repair was shown to be insulin dependent because the deficient osseous healing was reversed by insulin treatment (9). This finding demonstrates a specific cause and effect relationship between inadequate insulin production and abnormal bone formation. The production of matrix proteins was also diminished as demonstrated by findings that the fracture callus in diabetic animals exhibited a 60–70% reduction in the production of type X collagen during the endochondral period of bone formation, compared with normal animals (6). A number of mechanisms have been proposed for bone abnormalities in diabetics. Diminished expression of IGF-1 or basic fibroblast growth factor may contribute to reduced production of bone matrix, and the increased brittleness of diabetic bone may be due to abnormalities in microarchitecture (10–13).

Diabetes can be induced in mice by multiple low doses of streptozotocin (MLD-STZ). This is an improvement over the high-dose streptozotocin model because it more closely replicates the cellular events involved in the destruction of the insulin-producing cells in the pancreas and altered glucose transporter 2 expression (14). Although there is a component of mild toxicity of streptozotocin to pancreatic β-cells, reports indicate that activation of the immune system plays a central role in the mechanism by which MLD-STZ treatment results in destruction of pancreatic β-cells (15, 16). The resulting diabetes therefore has many similarities to type 1 human diabetes and is characterized by mild to moderate hyperglycemia, glucosuria, polyphagia, hypoinsulinemia, hyperlipidemia, and weight loss. Streptozotocin-induced diabetic animals also exhibit many of the complications observed in human diabetes including enhanced susceptibility to infection and cardiovascular disease, retinopathy, alterations in angiogenesis, delayed wound healing, diminished growth factor expression, and reduced bone formation (16, 17). These studies indicate that streptozotocin-induced diabetes in mice serve as a useful model to study mechanisms related to wound healing in general and osseous healing specifically. Most significantly, both MLD-STZ-induced diabetes and human type 1 diabetes develop similar features of osteopenia.

Bone formation can be investigated in a number of different model systems. Recently, the marrow ablation model has been used to investigate mechanisms by which growth factors and differentiation factors modulate intramembranous bone formation (18–20). In this model the marrow

Abbreviations: Cbfa1, Core-binding factor α1; Dlx5, human homolog of the *drosophila* distal-less gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MLD-STZ, multiple low doses of streptozotocin; RNase, ribonuclease; Runx-2, runt domain factor-2.

cavity of the diaphyseal portion of the tibia is disrupted by gently removing trabecular bone and marrow. Over a 10-d period, there is a predictable and synchronized series of events that include the removal of the hematoma that forms immediately after ablation; formation of immature mesenchymal tissue; differentiation of osteoblasts; and production of a bone matrix, which peaks on d 6. At 10 d and beyond, bone resorption occurs so that the marrow is reconstituted to its original form. During this later period of remodeling, coupled bone formation occurs, thus providing an excellent model system to study both nascent bone formation in response to injury and formation during a resorptive cycle. This system, unlike fracture repair, however, does not involve an endochondral component.

Materials and Methods

CD-1 mice (Charles River Laboratories, Inc., Wilmington, MA) were treated with streptozotocin (40 μg/1 g body weight) in 10 mM citrate buffer or 10 mM citrate buffer alone by ip injection daily for 5 d. Mice were considered to be diabetic when blood glucose levels exceeded 250 mg/dl. Marrow ablation of the tibia was performed after mice were diabetic for 12–14 d.

Insulin treatment

Additional experiments were carried out in streptozotocin-induced diabetic mice with and without insulin treatment. Insulin treatment was initiated when blood glucose levels exceeded 200 mg/dl. Mice were treated with Linbit insulin implants (LinShin Canada, Inc., Scarborough, Ontario, Canada) that release a controlled amount of insulin or identical pellets without insulin. Insulin-treated animals had blood glucose levels that were generally 100 mg/dl after insertion of insulin implants. Marrow ablation was then performed as described above when the diabetic group had glucose levels that exceeded 250 mg/dl for 2 wk. Blood glycosylated hemoglobin levels were measured at the time of death using a Glyco-Affinn GHb test (Perkin-Elmer Life Sciences, Norton, OH).

Marrow ablation of the tibia

An oblique incision was made over the patellar ligament and tibial tuberosity of the right and left leg. The tibial tuberosity through patellar ligament was punctured with a small endodontic file (Dentsply International, York, PA). The marrow and trabecular bone was gently removed by careful filing, and the marrow cavity was rinsed with sterile saline. The incision was closed with 5–0 silk sutures. At the indicated time points, mice were euthanized by CO_2 inhalation. One tibia from each animal was randomly assigned to histologic analysis and the other for mRNA analysis.

Histology

At the time of euthanasia, the tibia and distal femur were fixed in 4% paraformaldehyde in PBS at 4 C. Specimens were decalcified in Immunocal (Decal Chemical Corp., Congers, NY) and embedded in paraffin. Sagittal 5- to 6-μm sections were cut and stained with hematoxylin and eosin. Histologic analysis was carried out on sections from three to six animals per data point. Measurements were made starting approximately 1 mm from the patellar surface and included five to seven fields at magnification of $\times$20. The percent area of each field containing mesenchymal connective tissue, bone matrix, and bone marrow was assessed. One-way ANOVA was performed to establish statistically significant differences between the experimental and control groups at the P less than 0.05 level.

RNA analysis

At the time of euthanasia, the tibial bone was harvested from the proximal metaphysis to the tibiofibular junction excluding all cartilaginous and soft tissues. Tibiae were snap frozen in liquid nitrogen, pulverized, and three to six specimens combined for each ribonuclease (RNase) protection assay. Total RNA was extracted with Trizol (Life Technologies, Inc., Rockville, MD), and the concentration and integrity of the extracted RNA was verified by denaturing agarose gel electrophoresis. Gene expression was measured by the RNase protection assay. ^{32}P-Labeled riboprobes were incubated with 2 μg total RNA for matrix gene expression and 12 μg for transcription factor gene expression. Samples were then subjected to RNase digestion using a kit from PharMingen (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. Following electrophoresis on a 6% polyacrylamide gel, radiolabeled bands were visualized with a PhosphorImager (BioRad Laboratories, Hercules, CA). The OD of the protected bands was measured with Image ProPlus software (Media Cybernetics, Silver Spring, MD), which was then normalized by the value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same lane. To show changes relative to baseline, the value for the zero time point was subtracted from each of the subsequent time points. Two to four separate RNase protection assays were performed for expression of each gene with similar results.

Results

The initial event after marrow ablation was hematoma formation, which was present throughout the area of the marrow ablation on d 2 (Fig. 1). The cells of the hematoma became necrotic or were replaced by immature mesenchymal tissue by d 4. By d 6 much of the immature mesenchymal tissue was replaced by newly formed bone. Between d 6 and d 10, the formation of marrow elements was initiated, and the area of new bone matrix was reduced. Remodeling of the bone marrow continued from d 10–16.

Quantitative histomorphometric analysis was performed to measure the amount of connective tissue, bone and bone marrow present in both the diabetic and control groups (Fig. 2). Immature mesenchymal tissue first appeared in significant amounts on d 4 and an equivalent amount was present in the normal and diabetic groups. By d 6 abundant formation of new bone was seen in the normal group. In contrast, significantly reduced quantities of bone had formed in the diabetic group. Bone formation was followed by the appearance of bone marrow on d 10 and d 16. It is interesting to note that the reestablishment of the marrow was more advanced on d 10 and 16 in the control, compared with the diabetic group. By d 16, equal amounts of marrow were present in both groups.

To establish mechanisms to explain deficient formation of new bone in diabetic mice, RNase protection assays were carried out to quantify mRNA levels of genes encoding bone and stromal connective tissue matrix molecules. In addition, a number of transcription factors that are known to regulate bone formation were separately assayed.

Osteocalcin is a bone-specific matrix protein. Its expression was strongly induced on d 4 and 6 in the control mice and to a much lesser extent in diabetic animals (Fig. 3A). Moreover, the difference between the two groups was in the level of expression rather than the time frame in which expression occurred. For collagen type 1 mRNA, high levels in the control group were detected on d 2, peaked on d 4, and were expressed at lower levels on d 6 and 10 (Fig. 3B). The largest difference between the diabetic and normal group occurred on d 4.

The conversion of mesenchymal tissue to bone requires the differentiation of immature cells to osteoblasts. To examine

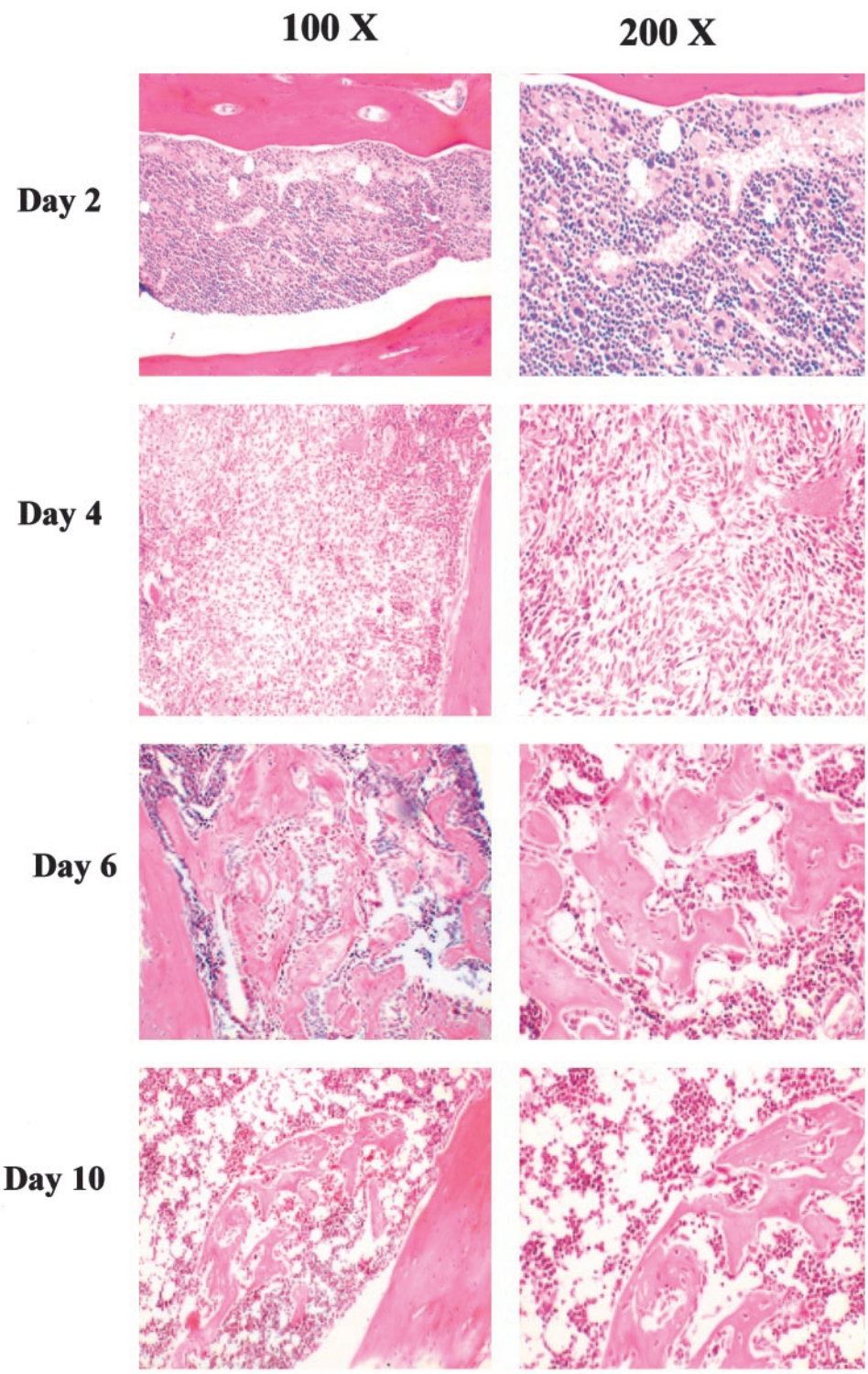

FIG. 1. Histologic changes following marrow ablation. Marrow ablation was performed in normal mice as described in *Materials and Methods*. Mice were killed at the indicated time points. Paraffin-embedded sections were stained with hematoxylin and eosin. Original magnification, ×100 *(left panels)* or ×200 *(right panels)*.

this process, we measured the expression of two transcription factors, Dlx5 and core-binding factor α1 (Cbfa1)/runt domain factor-2 (Runx-2), which are thought to control acquisition of an osteoblast phenotype (21–24). In the normal group, Dlx5 expression was high on d 4 and 6, consistent with the conversion of mesenchymal tissue to bone observed histologically during this period (Fig. 4A). In the diabetic mice, a peak in their expression did not occur so that the level of expression was suppressed during this critical period. Lower levels of expression were noted in both the control and experimental groups on d 10 and 16, which likely reflects formation associated with remodeling. Virtually the same pattern was observed for Cbfa1/Runx-2 m RNA levels (Fig. 4B). On d 4 and 6, Cbfa1/Runx-2 expression was high in the control group, but it was much lower in the diabetic animals.

The expression of protooncogenes c-fos and c-jun is asso-

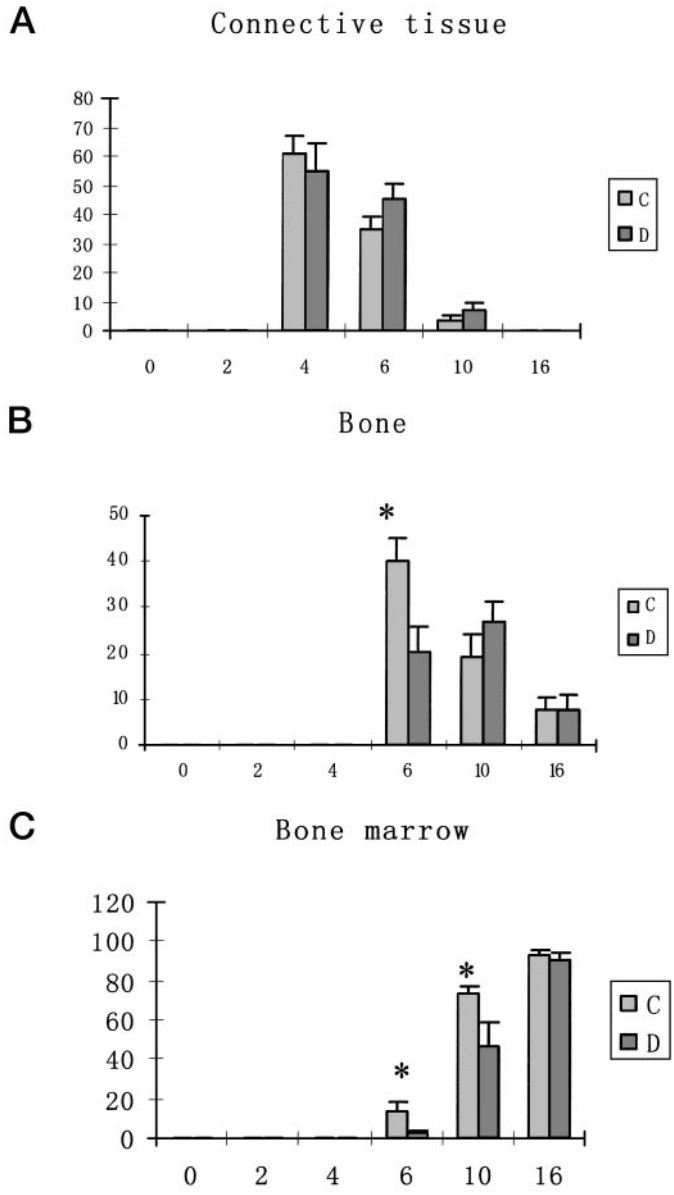

FIG. 2. Histomorphometric analysis of marrow ablation specimens. Marrow ablation was performed on diabetic or control mice, and sections were prepared as described in Fig. 1. The percent of each field containing connective tissue (A), bone (B), or bone marrow (C) was assessed at magnification of ×200. Each value represents the mean ± SEM. *, Values in which the control group was significantly different from the diabetic group ($P < 0.05$).

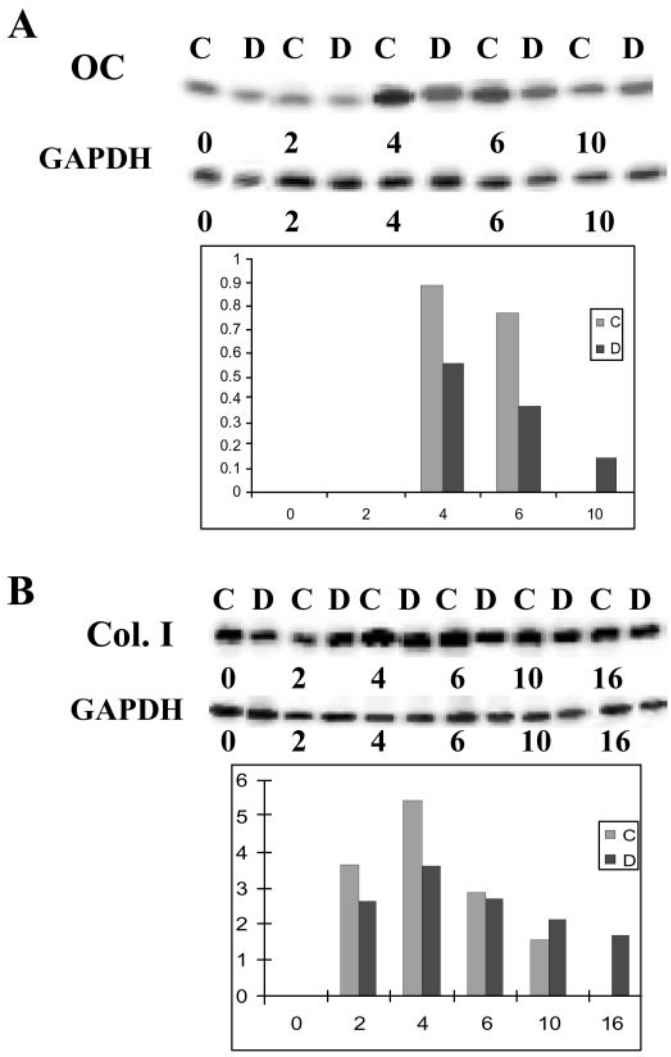

FIG. 3. Osteocalcin and collagen type 1 expression are reduced in diabetic mice during bone formation. Marrow ablation was performed on diabetic and normal mice as described in Fig. 1. The expression of osteocalcin and collagen type 1α1 was assessed at each time point by RNase protection assay. The densitometric value of each band was obtained using image analysis software and normalized by the value for GAPDH in the same lane. The PhosphorImage is shown with densitometric analysis below. A, Osteocalcin; B, collagen type 1.

ciated with a number of events that occur in bone including bone formation and hematopoiesis. We found that c-fos and c-jun expression was low in the normal mice on d 2, reached a peak on d 6, and progressively decreased on d 10 and 16 (Fig. 5). C-fos expression was similar in the control and diabetic groups at the early time points but in the diabetic group did not reach the same high levels on d 6 and 10 as the controls (Fig. 5A). In contrast, the levels of c-jun expression were not dramatically different between the experimental and control groups (Fig. 5B).

To examine the effect of insulin treatment on diabetic mice, the expression of bone matrix proteins osteocalcin and collagen type I and the transcription factors Dlx 5 and Cbfa1/Runx-2 were analyzed by ribonuclease protection assay. The insulin-treated mice had significantly higher expression of these genes, compared with the untreated control group (Figs. 6 and 7). The effectiveness of insulin treatment was evident by the much higher levels of glycosylated hemoglobin in the nontreated group, which ranged from 9.8% to 30.4% and a mean of 14.5%, compared with the insulin-treated mice that had range of 3.5–6.9% and a mean of 4.9%.

Discussion

The marrow ablation model is well suited to study the events of intramembranous bone formation, in part, because there is a synchronized progression of events from the formation of mesenchymal tissue followed by the formation of

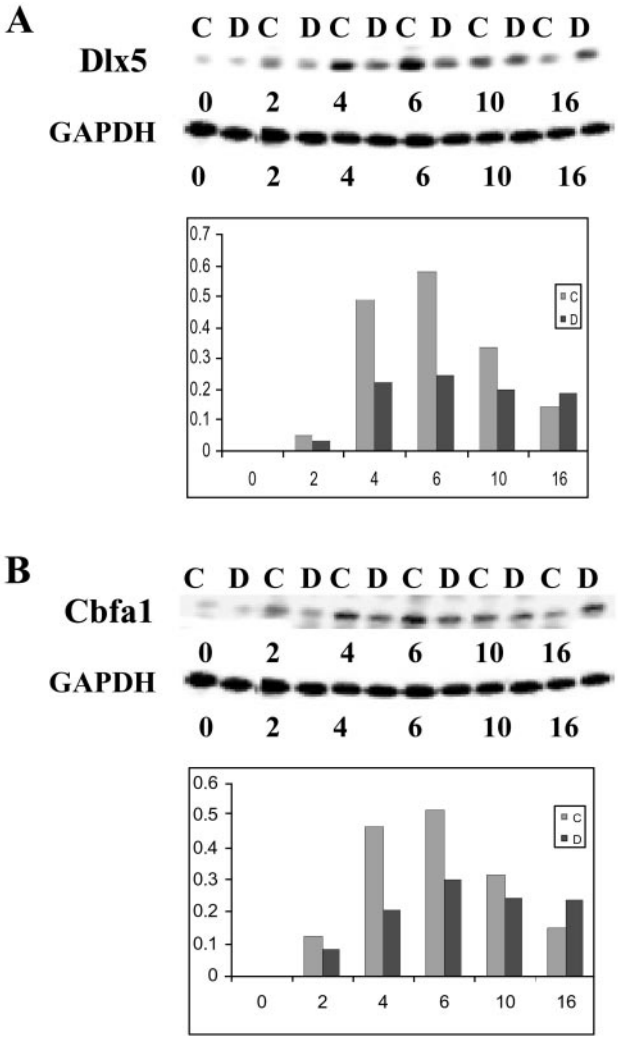

FIG. 4. The expression of transcription factors Dlx5 and Cbfa1/Runx-2 are reduced in diabetic mice during bone formation. Marrow ablation was performed on diabetic and normal mice as described in Fig. 1. The expression of Dlx5 and Cbfa1/Runx-2 were assessed at each time point by RNase protection assay. The densitometric value of each was obtained using image analysis software and normalized by the value for GAPDH in the same lane. The PhosphorImage is shown with densitometric analysis below. A, Dlx5; B, Cbfa1/Runx-2.

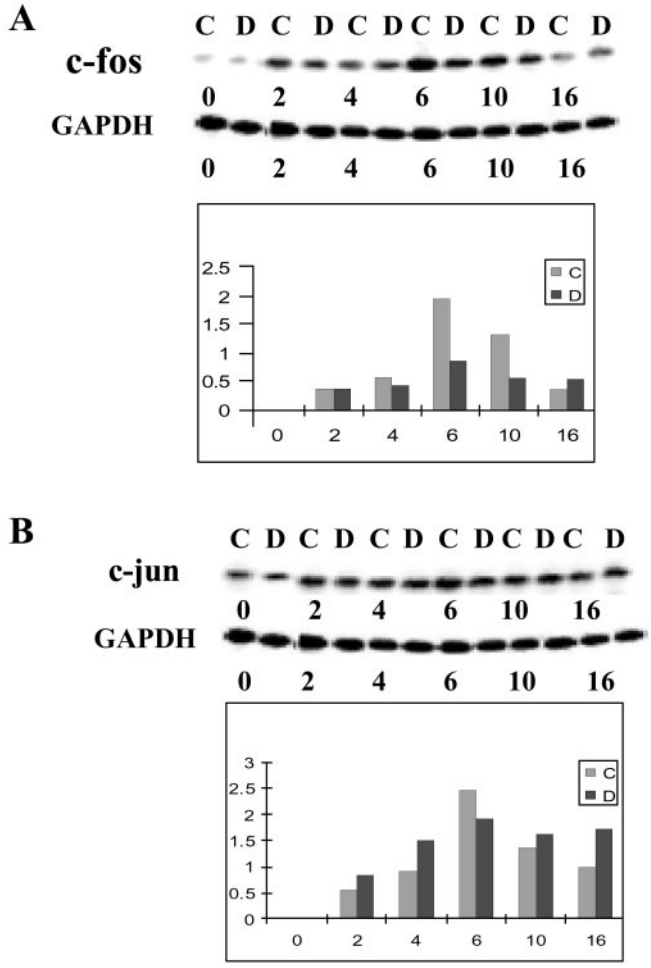

FIG. 5. The expression of c-fos and c-jun in diabetic and control mice during bone formation. Marrow ablation was performed on diabetic and normal mice as described in Fig. 1. The expression of c-fos and c-jun was assessed at each time point by RNase protection assay. The densitometric value of each was obtained using image analysis software and normalized by the value for GAPDH in the same lane. The PhosphorImage is shown with densitometric analysis below. A, c-fos; B, c-jun.

bone matrix and eventual reconstitution of the bone marrow. This synchronization facilitates comparison of cellular events at the histologic and molecular level. Consistent with other reports, histomorphometric analysis revealed a burst of bone formation on d 6 (18, 20, 25). This was preceded by the expression of transcription factors that regulate the osteoblast phenotype and the expression of matrix proteins. In contrast to control animals, the diabetic mice failed to exhibit an equivalent burst of osteoid production, representing formation of bone at the protein level. The diabetic group also had a substantially reduced expression of genes that encode bone matrix proteins. Consistent with this observation, the diabetic group also failed to express high levels of transcription factors that regulate osteoblast differentiation. Control experiments indicated that the reduced expression of matrix and osteoblast-regulating transcription factors observed in diabetic mice is due to the diabetic state rather than a side effect of streptozotocin treatment because it was reversed by insulin treatment. Previous investigators have also reported that osteopenia associated with streptozotocin-induced diabetes is reversed by insulin treatment (9).

Of the matrix proteins examined, osteocalcin is specifically restricted to bone. In the control group, its expression occurred above baseline levels on d 4 and 6. Expression on d 4 represents a point at which osteoblast differentiation has occurred, but osteoid matrix has not yet accumulated. On d 6 there is abundant bone matrix present and the continued expression of osteocalcin. The difference between the expression of osteocalcin in the diabetic and control groups was striking. The levels in the diabetic group were substantially reduced, but there was no delay in the onset of osteocalcin expression.

Although diabetic mice produced less bone than their normal counterparts, they did produce a similar amount of immature mesenchymal tissue. When the expression of Dlx5

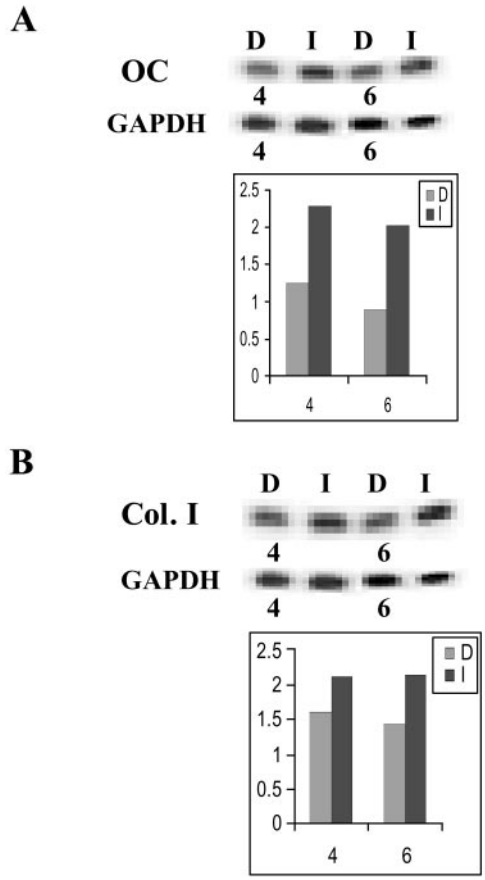

FIG. 6. The decrease in osteocalcin and collagen type 1 expression in diabetic mice is reversed with insulin treatment during bone formation. Marrow ablation was performed on insulin-treated and nontreated diabetic mice as described in Fig. 1. The expression of osteocalcin and collagen type 1α1 was assessed at each time point by RNase protection assay. The densitometric value of each was obtained using image analysis software and was normalized by the value for GAPDH in the same lane. The PhosphorImage is shown with densitometric analysis *below*. A, Osteocalcin; B, collagen type 1.

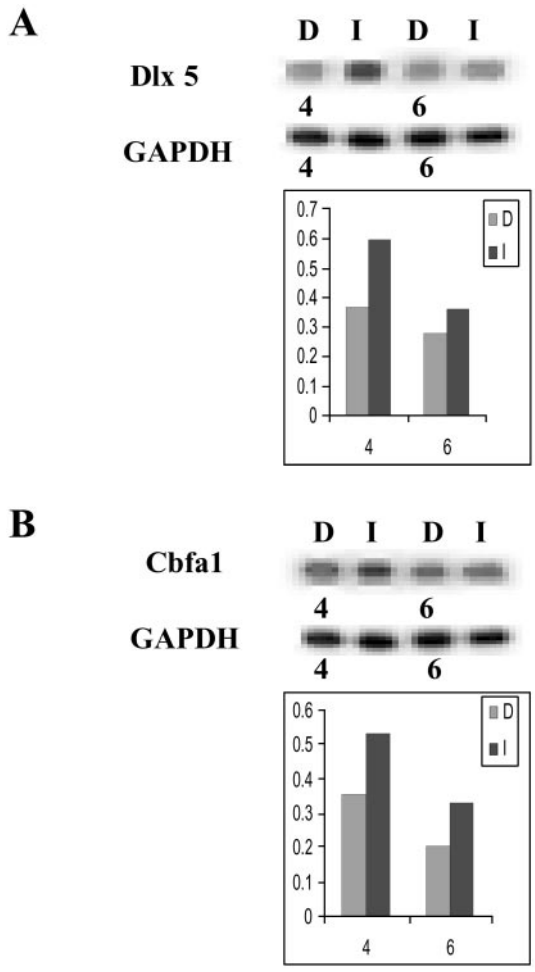

FIG. 7. The decrease in transcription factors Dlx5 and Cbfa1/Runx-2 expression in diabetic mice is reversed with insulin treatment during bone formation. Marrow ablation was performed on insulin-treated and nontreated diabetic mice as described in Fig. 1. The expression of Dlx5 and Cbfa1/Runx-2 were assessed at each time point by RNase protection assay. The densitometric value of each was obtained using image analysis software and was normalized by the value for GAPDH in the same lane. The PhosphorImage is shown with densitometric analysis *below*. A, Dlx5; B, Cbfa1/Runx-2.

and Cbfa1/Runx-2 were examined, high levels were induced on d 4 in the control but not the diabetic group. Given the importance of Cbfa1/Runx-2 in osteoblast differentiation, its diminished expression in the diabetic group is likely to be a critical event in explaining the reduced formation of bone observed histologically. Thus, diabetic individuals may have reduced Cbfa1/Runx-2 expression that may lead to a reduction in osteoblast differentiation, which in turn may reduce the expression of bone-matrix genes and limit production of new bone. It is striking that the expression of these osteoblast-regulating transcription factors was not delayed so much as diminished in the diabetic state. At later time points (d 10 and 16), Cbfa1/Runx-2 expression was clearly detected in both the control and experimental groups, although at lower levels than those found during peak bone formation. This likely is due to bone formation associated with remodeling that occurs beyond d 6 in the ablation model.

Collagen type I is found in immature mesenchymal tissue, the stromal lining of bone and bone matrix. Collagen type 1 was expressed at relatively high levels from d 2 to 10 reflecting its expression in these various tissues. A similar temporal pattern of collagen expression has been previously reported following marrow ablation of rat tibia (18). The control and diabetic groups exhibited a substantial difference in the level of mRNA for collagen type I on the day that it peaked in the wild-type group. Thus, diabetic animals failed to exhibit the same burst of collagen type 1 gene expression. Interestingly, in a similar marrow ablation model, aged rats experienced reduced but not delayed expression of osteocalcin, collagen type 1 and IGF-1, compared with young rats (25, 26). This suggests that there are phenotypic similarities in diminished bone production associated with aging and diabetes.

In the ablation model, the proliferation of mesenchymal cells occurs between d 2 and 4, and the reconstitution of marrow elements and hematopoiesis is initiated by d 6 and continues to d 16. Both c-fos and c-jun form dimers and are members of the activator protein-1 family of transcriptional regulators. These transcription factors are expressed at high

levels during proliferation of preosteoblastic cells during the early stages of osteoblast differentiation, induced by bone morphogenetic proteins, and involved in hematopoiesis (27–29). The similarity of c-jun and c-fos expression in diabetic and control mice at d 2 and 4 are consistent with the equal production of immature mesenchymal tissue containing preosteoblasts. However, on d 6, c-fos expression was considerably higher in the control than diabetic group, but this difference was not observed for c-jun. Thus, c-fos expression may be related to the production of bone matrix.

In summary, the osseous healing that takes place following marrow ablation results in a burst of cellular activity that involves the induction of transcription factors that regulate osteoblast differentiation, the expression of matrix proteins and the formation of an organized matrix. In diabetic mice the sharp up-regulation of Dlx5 and Cbfa1/Runx-2 is attenuated with the results being a deficiency in conversion of immature mesenchymal cells to mature osteoblasts. This agrees well with the histologic evidence for diminished bone formation during this period.

Acknowledgments

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