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Diabetes causes the accelerated loss of cartilage during fracture repair which is reversed by insulin treatment

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ABSTRACT

Fracture healing in diabetic individuals and in animal models of diabetes is impaired. To investigate mechanisms by which diabetes may affect fracture healing we focused on the transition from cartilage to bone, a midpoint in the fracture healing process. Femoral fractures were induced in mice rendered diabetic by multiple low dose streptozotocin treatment and compared to matching normoglycemic mice. One group of diabetic animals was treated with slow release insulin to maintain normal serum glucose levels. The results indicate that there was relatively little difference in the initial formation of the fracture callus on day 10. However, on day 16 the diabetic group had significantly smaller callus, greater loss of cartilage and enhanced osteoclastogenesis that was normalized by treatment with insulin when assessed by histomorphometric analysis. Chondrocyte apoptosis was significantly higher in diabetic mice and this increase was blocked by insulin. These changes were accompanied by diabetes-increased mRNA levels of RANKL, TNF-α, and ADAMTS-4 and -5 measured by real-time PCR, which was reversed by insulin treatment. On days 16 and 22 bone formation within the callus of diabetic mice was significantly less than the normoglycemic and brought to normal levels by insulin treatment. These results suggest that a significant effect of diabetes on fracture healing is increased chondrocyte apoptosis and osteoclastogenesis that accelerates the loss of cartilage and reduces the anlage for endochondral bone formation during fracture repair. That insulin reverses these effects demonstrates that they are directly related to the diabetic condition.

Introduction

Fracture healing is a complicated multi-phase process that involves the coordinated activity of many cell types [1]. The healing process is initiated by a hematoma that forms in response to the disruption of blood vessels. Progenitors are then recruited to the site of injury where they proliferate and differentiate into chondrocytes and osteoblasts. Chondrocytes produce cartilage forming a soft cartilaginous callus, which calcifies and protects the injured site. As chondrocytes undergo apoptosis osteoclasts begin the removal of mineralized cartilage, setting the stage for endochondral bone formation by osteoblasts. The bony callus then undergoes remodeling until the bone reaches it original form.

Bone is affected by diabetes, which causes osteopenia and impairs fracture healing [2,3]. Osteopenia is thought to be a contributing factor to the increased fracture risk observed in diabetic patients [4–6]. Most studies on osteopenia have focused on impaired bone formation, which is supported by a decrease in bone mineral density and reduced markers of bone formation such as serum levels of osteocalcin and alkaline phosphatase [7–9]. Several mechanisms have been suggested including changes in cell signaling caused by hyperglycemia, inflammation associated with diabetes, changes in circulating growth factors and endocrine hormones, greater oxidative stress and increased cell death [2,8,10]. Krakauer et al. have suggested that patients with diabetes have reduced bone formation and bone accumulation during growth, while later in life hyperglycemia leads to increased bone resorption and osteopenia [11]. Recent evidence supports the concept that diabetes can contribute to osteopenia by increasing osteoclast formation [11–14].

Case reports and clinical investigations have reported that diabetes delays union of healing fractures and increases healing time in diabetic subjects compared to matched controls [5,15,16]. Animal models also demonstrate that diabetes leads to the formation of smaller calluses with decreased bone and cartilage formation, decreased proliferation and differentiation of osteoblastic cells and chondrocytes and a two-fold reduction in the mechanical strength during fracture repair in diabetic compared

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Table 1
Serum glucose and glycosylated hemoglobin values

	Normoglycemic	Diabetic	Diabetic+insulin
Serum glucose	147±3.2	490±12.9	108±6.4
Glycosylated hemoglobin	6.2±0.18	12.2±0.24	7.4±0.25

Serum glucose levels were measured weekly starting two weeks after induction of diabetes and the mean values are given for each group. Glycosylated hemoglobin levels were measured at the time of euthanasia from blood obtained by cardiac puncture.

to normoglycemic animals [17–21]. DNA content is decreased by 40% in healing diabetic fractures compared to controls, an indication that the diabetic calluses have decreased cellularity [22]. This could be due a decrease in the rate of cell proliferation associated with decreased growth factor production [23]. In addition, there is a decrease in the collagen content of the callus of the diabetic animals compared with normoglycemic animals [19,22]. A decrease in matrix could results from reduced formation of osteoblasts that produce bone [24].

We previously investigated the impact of diabetes on fracture healing in the tibia. The results identified a previously unrecognized catabolic effect of diabetes on fracture repair, the accelerated loss of cartilage in the diabetic group [25]. To investigate further the impact of diabetes on endochondral bone formation experiments were carried out where fractures were induced in the femur and the impact of diabetes was tested by treating mice with slow release insulin. Histologic and molecular analysis indicated that diabetes caused an increased osteoclastogenesis and loss of cartilage and increased mRNA levels of several pro-resorptive factors. Each of these parameters was reversed by treatment with insulin. These studies represent an important extension of our previous results since they demonstrate that the catabolic events are specifically related to the diabetic state since they are rescued by insulin treatment.

Materials and methods

Induction of type 1 diabetes

All experiments were approved by the Boston University Medical Center Institutional Animal Care and Use Committee (IACUC). Eight week old, male CD-1 mice purchased from Charles River Laboratories (Wilmington, MA) were rendered diabetic by intraperitoneal injections (i.p.) of streptozotocin (40 mg/kg) (Sigma, St. Louis, MO) in 10 mM citrate buffer daily for 5 days [26]. Control mice were treated identically with vehicle alone, 10 mM citrate buffer. A group of diabetic mice received insulin treatment through slow release insulin implants (Linbit, Linshin Canada, Toronto, ON) placed subcutaneously. These implants release ~0.1 U of insulin per day and depending on the weight of the animal, each animal received 4 to 5 implant according to the manufacturer's instructions. Animals were considered to be diabetic when serum glucose levels exceeded 250 mg/dl (Accu-Chek, Roche Diagnostics, Indianapolis, IN) and were measured weekly. Glycosylated hemoglobin levels were measured at the time of euthanasia by Glyco-tek affinity chromatography (Helena Laboratories, Beaumont, TX) (Table 1).

Femoral fractures

All studies were performed on male mice that were diabetic for 3 weeks prior to fracture. A simple transverse closed fracture of the femur was performed in separate animals as previously described [1,25,27]. In the femoral fractures, the incision was made lateral to the

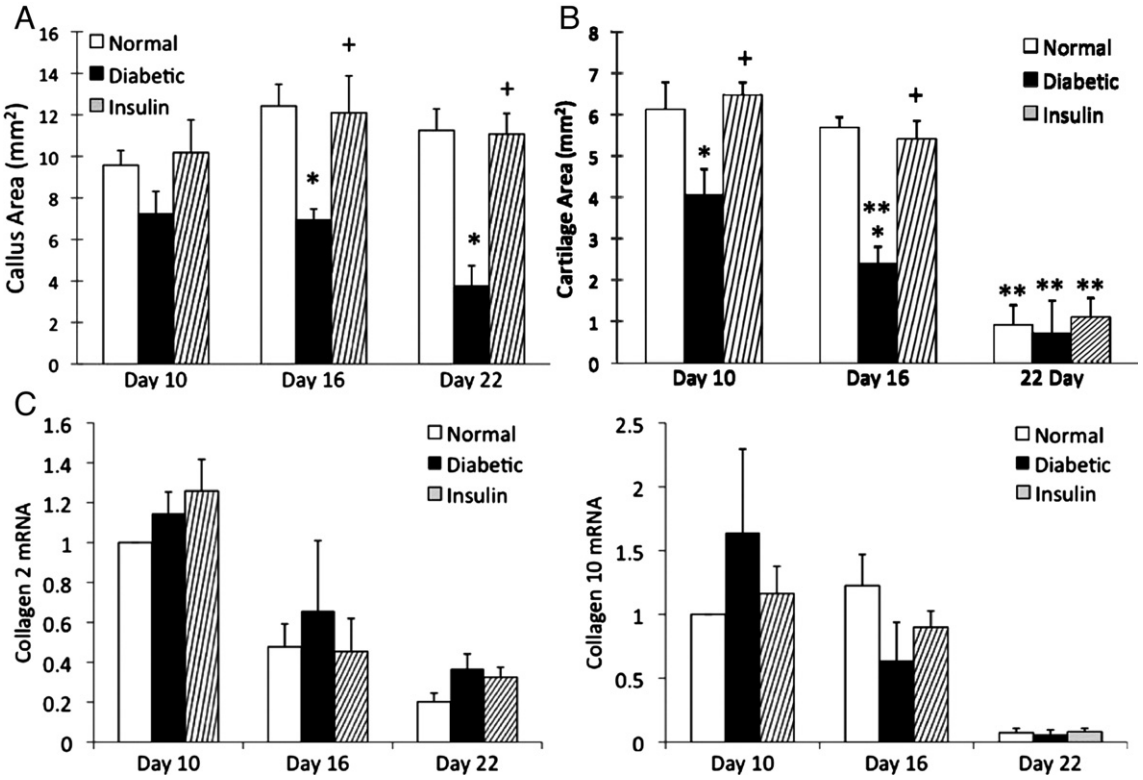

Fig. 1. (A) Comparison of callus size in diabetic, normoglycemic and diabetic insulin treated mice with femoral fracture. Callus area was measured in H&E stained cross-sections obtained from 3 points sampled at the fracture line and 0.5 mm proximal and distal and presented as the sum of these three sites. (B) Comparison of cartilage area in diabetic, normoglycemic and diabetic insulin treated mice with femoral fracture. The area of cartilage within each callus was measured in safranin-O/fast green stained sections in the same manner. The individual measurements were averaged to establish a value of total callus area per animal for each of the 3 time points examined. Data are expressed as mean±SEM. * indicates a significant difference between normal and diabetic ($P<0.05$). + indicates significant difference between insulin treated and untreated diabetic animals ($P<0.05$). ** indicates a significant difference compared with the previous time point within a group ($P<0.05$). (C) mRNA levels of markers of cartilage formation. mRNA levels were measured by real-time PCR for Collagen 2 and collagen 10. Each marker was evaluated in 3 separate experiments using 3 mice in each set (total $n=3$) and the results are expressed as mean±SEM.