Musculoskeletal Pathology

High Levels of Tumor Necrosis Factor- α Contribute to Accelerated Loss of Cartilage in Diabetic Fracture Healing

Jazia Alblowi,* Rayyan A. Kayal,* Michelle Siqueria,* Erin McKenzie,* Nanarao Krothapalli,* Jody McLean,[†] Jason Conn,* Barbara Nikolajczyk,[‡] Thomas A. Einhorn,[†] Louis Gerstenfeld,[†] and Dana T. Graves[§]

From the Department of Periodontology and Oral Biology,* Boston University School of Dental Medicine, Boston, Massachusetts; the Departments of Orthopedic Surgery[†] and Microbiology,[‡] Boston University School of Medicine, Boston, Massachusetts; and the Department of Periodontics,[§] University of Medicine and Dentistry, Newark, New Jersey

Diabetes interferes with fracture repair; therefore, we investigated mechanisms of impaired fracture healing in a model of multiple low-dose streptozotocin-induced diabetes. Microarray and gene set enrichment analysis revealed an up-regulation of gene sets related to inflammation, including tumor necrosis factor (TNF) signaling in the diabetic group, when cartilage is being replaced by bone on day 16, but not on days 12 or 22. This change coincided with elevated osteoclast numbers and accelerated removal of cartilage in the diabetic group ($P < 0.05$), which was reflected by smaller callus size. When diabetic mice were treated with the TNF-specific inhibitor, pegsunercept, the number of osteoclasts, cartilage loss, and number of TNF- α and receptor activator for nuclear factor κ B ligand positive chondrocytes were significantly reduced ($P < 0.05$). The transcription factor forkhead box 01 (FOXO1) was tested for mediating TNF stimulation of osteoclastogenic and inflammatory factors in bone morphogenetic protein 2 pretreated ATDC5 and C3H10T1/2 chondrogenic cells. FOXO1 knock-down by small-interfering RNA significantly reduced TNF- α , receptor activator for nuclear factor κ B ligand, macrophage colony-stimulating factor, interleukin-1 α , and interleukin-6 mRNA compared with scrambled small-interfering RNA. An association between FOXO1 and the TNF- α promoter was demonstrated by chromatin immunoprecipitation assay. Moreover, diabetes increased FOXO1 nuclear

translocation in chondrocytes *in vivo* and increased FOXO1 DNA binding activity in diabetic fracture calluses ($P < 0.05$). These results suggest that diabetes-enhanced TNF- α increases the expression of resorptive factors in chondrocytes through a process that involves activation of FOXO1 and that TNF- α dysregulation leads to enhanced osteoclast formation and accelerated loss of cartilage. (Am J Pathol 2009, 175:1574–1585; DOI: 10.2353/ajpath.2009.090148)

Osteopenia associated with decreased bone mineral density is an important complication of type 1 diabetes.^{1–5} The effect of osteopenia is thought to significantly enhance the risk of fractures as evidenced by increased fractures of the long bones of diabetics.^{6–8} Clinical studies have reported delayed union or increased fracture healing time in diabetic subjects compared with matched controls.^{9–11} Similar findings of impaired or delayed fracture healing have been reported in multiple animal models.^{12–14}

Normal fracture repair is dependent on the coordinated expression of cytokines that initiate and regulate the fracture healing process including the production and removal of cartilage coupled with bone formation and remodeling.¹⁵ Diabetes has been shown to enhance expression of receptor activator for nuclear factor κ B ligand (RANKL), macrophage colony-stimulating factor (M-CSF), and tumor necrosis factor- α (TNF- α) that stimulate formation of osteoclasts and are responsible for resorption of mineralized cartilage and bone.^{16–18} Under some conditions diabetes has been shown to increase osteoclastogenesis.^{18–22} Diabetes-enhanced osteoclast formation is thought to contribute to diabetic osteopenia in adults as well as in acute Charcot arthropathy, a compli-

Supported by NIH grants P01AR49920, R01DE07559, and R01DE017732.

Accepted for publication June 26, 2009.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

Address reprint requests to Dr. Dana T. Graves, University of Medicine and Dentistry, Department of Periodontics, 110 Bergen St, Room C-781, Newark, NJ 07111. E-mail: gravesdt@umdnj.edu.

cation of diabetic neuropathy that increases bone fragility and in diabetic fracture healing.^{2,23,24} In both conditions increased osteoclastogenesis is linked to increased expression of pro-resorptive factors including RANKL, M-CSF, and TNF- α .²⁴

One of the mechanisms by which diabetes may impair fracture healing is through increased levels of TNF- α .¹⁶ Increased TNF- α is thought to contribute to a number of diabetic complications including microangiopathy and neuropathy, cardiovascular diseases, retinopathy, and increased inflammation associated with infection and periodontitis.^{25,26} Although nuclear factor κ B is typically associated with TNF-induced inflammation,²⁷ it is also likely that other transcription factors play an important role. Because we previously demonstrated that forkhead box O1 (FOXO1) mediated the pro-apoptotic effects of TNF- α and TNF- α -induced pro-apoptotic gene expression,²⁸ the experiments described below were undertaken to determine whether TNF- α contributed to impaired fracture healing *in vivo* and whether FOXO1 could potentially regulate mRNA levels of pro-osteoclastogenic factors induced by TNF- α *in vitro*.

Forkhead transcription factors of the FOXO subfamily regulate metabolism, proliferation, and differentiation.²⁹ FOXO1 is activated by oxidative stress and induces the expression of genes that decrease reactive oxygen species, thereby protecting the cell from oxidative stress.³⁰ FOXO1 is up-regulated by TNF and has been shown to mediate the pro-apoptotic effects of TNF.²⁸ It has been proposed that conditions with prolonged or high levels of FOXO1 activation may be deleterious by inducing apoptosis.^{31,32}

We previously reported that diabetes accelerates loss of cartilage in fracture healing by increasing osteoclastogenesis and the expression of factors that promote bone resorption.¹⁶ To investigate a mechanism for these events we treated diabetic mice with the TNF-specific inhibitor, pegsunercept, and examined the impact on diabetes-impaired fracture healing. In addition a potential downstream mechanism, FOXO1-mediated gene expression was further investigated *in vitro*. The results indicate that TNF- α plays a significant role in diabetes-enhanced osteoclastogenesis and accelerated cartilage degradation *in vivo*. Moreover, TNF- α *in vitro* stimulated mRNA levels of factors in chondrocytic cells that were pro-osteoclastogenic or pro-inflammatory, which was mediated in part by FOXO1. These studies provide new insight into diabetes impaired fracture healing and support a previously unrecognized role for TNF- α and FOXO1 in mediating this untoward response.

Materials and Methods

Induction of Type 1 Diabetes

The research was conducted in conformity with all Federal and U.S. Department of Agriculture guidelines, as well as an Institutional Animal Care and Use Committee approved protocol. Studies were done on 8-week-old, male CD-1 mice purchased from Charles River Laboratories (Wilmington,

MA). Diabetes was induced by intraperitoneal injection of streptozotocin (40 mg/kg) (Sigma, St. Louis, MO) in 10 mmol/L citrate buffer daily for 5 days.³³ Normoglycemic control mice were treated with vehicle alone, 10 mmol/L citrate buffer. Venous blood obtained from the tail was assessed for glucose levels (Accu-Chek, Roche Diagnostics, Indianapolis, IN) and mice were considered to be diabetic when blood glucose levels exceeded 250 mg/dl. Glycosylated hemoglobin levels were measured by Glyco-tek affinity chromatography (Helena Laboratories, Beaumont, TX) at the time of euthanasia (Supplemental Table 1, see <http://ajp.amjpathol.org>). Treatment of a group of diabetic animals with the TNF- α inhibitor pegsunercept by intraperitoneal injection (4 mg/kg) every 3 days was performed starting on day 10 after fracture until the time for euthanasia. Treatment with pegsunercept resulted in no significant differences in glycated hemoglobin level ($P > 0.05$) (Supplemental Table 1, A and B, see <http://ajp.amjpathol.org>).

Tibial and Femoral Fracture

A simple transverse closed fracture of the tibia or femur was performed on male mice that were diabetic for at least 3 weeks beforehand as previously described.^{16,34,35} Briefly, a 27 gauge spinal needle was inserted for fixation into the marrow cavity of the long bone. After closure of the incision, a fracture was created by blunt trauma. Fractures that were not consistent with standardized placement criteria (mid-diaphyseal) or grossly comminuted were excluded. For tibial fractures, days 12, 16, and 22 were examined and for femoral fractures, days 10 and 16 were studied. Day 12 in the tibial and day 10 in the femoral fracture are roughly equivalent time points in each when collagen is the predominant tissue in the fracture callus.^{16,33} Animals were euthanized at day 12, 16, and 22 time points for the mRNA profiling and day 10 and 16 time points for all other analysis. Tibias or femurs were harvested and the soft tissue was carefully removed.

mRNA Profiling of Gene Sets that Regulate Inflammation

After euthanasia, tibial fracture calluses from 12, 16, and 22 day groups were carefully dissected by removing all muscle and non-callus tissue and were immediately frozen in liquid nitrogen. Total RNA was extracted with Trizol (Life Technologies, Rockville, MD) from pulverized frozen tissue and further purified by an RNeasy MinElute cleanup kit (Qiagen, Valencia, CA). The concentration and integrity of the extracted RNA was verified by 260 nm/280 nm spectrophotometry and denaturing agarose gel electrophoresis with ethidium bromide staining. mRNA profiling was performed by using a PGA Mouse v1.1 array. Microarray probe preparation, hybridization, and reading of fluorescent intensity were performed by the Massachusetts General Hospital Microarray Core Facility (Cambridge, MA). All slides were co-printed with an internal "alien" sequence that has no sequence homologues in the mouse genome. Slide printing, array labeling, hybridization, and slide reading were performed at

the Massachusetts General Hospital Genomics Core Facility as previously described.³⁶ All slides were quality control tested and contained appropriate positive and negative control sequences for data analysis. The alien gene in these studies serves as both a genome-extrinsic sequence and serves as a universal in-spot reference. In the experiments reported here all microarrays were printed with an alien 70 mer probe that was co-printed with each gene specific probe such that the alien was at a final concentration of 10% of the murine gene oligonucleotide. The data were normalized by scaling all individual intensities such that the mean total intensities were the same for all comparative samples within a single array and across replicates. Using the intensity reading, background was calculated locally per spot and subtracted from the intensity measurement of each hybridized spot. The ratio of normal over alien was first calculated. Using this ratio, all outliers for each gene were discarded. The standard $\log_{10}$ of diabetic versus normal and diabetic versus alien for each spot was calculated and the distribution of the log ratios was obtained from combined replicates per time point. The data are combined using the geometric mean of four replicate ratios. Microarray data were analyzed by using gene set enrichment analysis (GSEA) described in Subramanian et al.³⁷ Genes were first ranked based on the correlation between their expression and the class distinction. An enrichment score was then calculated, which reflects the degree to which a gene is over-represented at the extremes (top or bottom) of the entire ranked list. Statistical significance (P value) and false discovery rate (FDR) of that enrichment score was then calculated. This statistical analysis determines whether a gene set is significantly up-regulated or down-regulated in the experimental sample compared with a control. Gene sets with a FDR less than 25% and a nominal P value less than 0.05 were considered significant.³⁷ Genes that contributed to a significant difference in pathways related to inflammation were identified by the GSEA software and further screened for significance by Student's t -test ($P \leq 0.05$) and for a threshold change of 1.5 increase or decrease in the diabetic versus normoglycemic microarray values.

For selected genes mRNA levels obtained with microarrays were validated by real-time PCR by using primers and probe sets purchased from Applied Biosystems (Foster City, CA). For a given experiment RNA from six to nine specimens were combined and TaqMan reagents were used for first-strand cDNA synthesis and amplification. Results were normalized with an 18S ribosomal primer and probe set. Each experiment was performed three times and the results from the three separate experiments were combined to derive mean values. The level of a given gene was set relative to the value obtained for the normoglycemic control animals on day 12.

Histology and Histomorphometric Analysis

Femoral samples from day 10 and 16 groups were fixed for 72 hours in cold 4% paraformaldehyde and decalcified for 2 weeks by incubation in cold Immunocal (Decal Corpora-

tion, Congers, NY). The internal fixation pin was removed after decalcification and femurs were embedded in paraffin, sectioned at 5 μ m and histomorphometric measurements were performed as described in Gerstenfeld et al.³⁴ Sections stained with safranin-O/fast green and H&E were used to measure cartilage area and callus size, respectively, by computer assisted image analysis by using Image ProPlus software (Media Cybernetics, Silver Spring, MD) as previously described.³⁸ The number of multinucleated, tartrate-resistant acid phosphatase positive cells lining bone or cartilage was counted to assess osteoclasts.³⁸ There were six to eight specimens per group and measurements were made blindly by one examiner, with the results confirmed by a second examiner.

Immunohistochemistry

Sections from formalin-fixed, paraffin-embedded samples were deparaffinized and antigen retrieval was performed by immersion in 10 mmol/L sodium citrate (pH 6.0) for 5 minutes at 95°C. Specimens were quenched with 3% hydrogen peroxide and blocked with avidin-biotin (Vector Laboratories, Burlingame, CA) and nonimmune serum matching the secondary antibody. Sections were incubated with primary antibodies at 4°C degree overnight, followed by rinsing and incubation with biotin labeled secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Goat polyclonal anti-murine TNF- α and anti-RANKL antibodies as well as matched control IgG were purchased from Santa Cruz Biotechnology. An additional negative control was performed consisting of primary antibody incubated with excess blocking peptide. Immune complexes were localized with an avidin-biotin horseradish peroxidase kit from Vector Laboratories, visualized with the chromogen 3, 3'-diaminobenzidine (Zymed Laboratories, Inc., South San Francisco, CA) and counterstained with hematoxylin. Analysis of the cartilage was performed by comparison with safranin-O/fast green stained serial sections and the number of positive proliferative or hypertrophic zone chondrocytes were counted at $\times 1000$ magnification and expressed as the percentage that were immunopositive. The expression of genes of interest in osteoblasts/mesenchymal cells in newly forming bone, fibroblasts in the callus capsule, and hypertrophic and proliferative chondrocytes was also assessed by using a scale from 0 to 5. This analysis was performed at $\times 1000$ magnification in a minimum of 20 fields per callus by using the following scale: 0 = no immunopositive cells; 1 = $<10\%$ of the cells of interest were lightly immunostained; 2 = $<10\%$ moderate to darkly immunostained; 3 = 10 to 25% moderate to darkly immunostained; 4 = 25 to 40% moderate to darkly immunostained; and 5 = greater than 40% of the cells of interest were darkly immunostained. For each data point $n = 6$ per group. Analysis was done blindly by one examiner with the results confirmed by a second blinded examiner.

FOXO1 Nuclear Translocation

Sections were prepared as described above and incubated overnight with anti-FOXO1 antibody (Santa Cruz

Biotechnology) or matched negative control antibody. Primary antibody to FOXO1 was detected by a Cy5 tagged secondary antibody. Propidium iodide nuclear stain was included in the mounting media. FOXO1 nuclear translocation was detected by confocal laser scanning microscopy at a focal plane that bisected the nuclei (Axiovert-100M, Carl Zeiss, Thornwood, NY). Cy5, propidium iodide, and phase contrast images (original magnification, $\times 400$) of the same field were digitally captured. The entire cartilage area in nonoverlapping fields was analyzed and the percentage of chondrocytes with unambiguous nuclear translocation was assessed by comparing FOXO1/Cy5, propidium iodide, merged and phase contrast images. For each group $n =$ six to eight specimens. Results were confirmed by a second examiner.

Cell Culture

In vitro experiments were performed with murine ATDC5 chondrogenic and C3H10T1/2 mesenchymal stem cells that were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Cellgro, Manassas, VA) supplemented with 5% or 10% fetal bovine serum, respectively, and 1% penicillin/streptomycin. To induce chondrocyte differentiation cells were cultured in media supplemented with 0.5% fetal bovine serum and 200 ng/ml bone morphogenetic protein 2 (BMP2) (Peprotech, Rocky Hill, NJ) for 6 days in the case of ATDC5 and 100 ng/ml BMP-2 for 4 days for C3H10T1/2 cells as described in Shea et al.³⁹ and Denker et al.⁴⁰

FOXO1 RNA Interference Studies

ATDC5 and C3H10T1/2 cells were plated in 6 well plates and when they were 70% confluent they were transfected with FOXO1 small-interfering RNA (siRNA) (5 nmol/L) or scrambled siRNA (Qiagen) with HiPerfect (Qiagen) in media supplemented with fetal bovine serum (0.25%) for 24 hours. The target sequence of FOXO siRNA was 5'-CCAGCTATAAATGGACATTTA-3'. After transfection for 24 hours, media was changed and cells were incubated for another 24 hours and then stimulated with 20 ng/ml of murine TNF- α (Peprotech) for the indicated period of time. Total RNA was extracted by using QIA shredder Mini Spin Columns and RNeasy Mini Kit (Qiagen). cDNA was prepared by using MultiScribe Reverse Transcriptase (Applied Biosystems) and real-time PCR was performed by using TaqMan primers and probe sets (Applied Biosystems). Results were normalized with ribosomal protein L32 (Applied Biosystems). In some experiments, nuclear proteins were extracted and FOXO1 DNA binding activity was measured as described below.

FOXO1 DNA Binding Activity

Nuclear and cytoplasmic proteins were extracted from cell lysates or from frozen pulverized trimmed femoral fracture calluses by using NE-PER Nuclear and Cytoplasmic Extraction Reagents kits (Pierce, Rockford, IL) supple-

mented with a protease inhibitor cocktail (Pierce). Protein content of the nuclear and cytoplasmic extracts was measured by bicinchoninic acid protein assay (Pierce). FOXO1 DNA binding activity was measured by using a transcription factor enzyme-linked immunosorbent assay (ELISA) for FOXO1 (Active Motif, Carlsbad, CA). TNF- α protein levels were measured by ELISA (R&D, Minneapolis, MN).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays (ChIPs) were performed by using a ChIP it Kit (Active Motif) with $\sim 1.5 \times 10^7$ ATDC5 murine chondrogenic cells per group, which were pretreated with BMP-2 (Peprotech) for 6 days. Cells at 70 to 80% confluency were stimulated with TNF- α for 30 or 60 minutes and compared with unstimulated cells. ChIP was performed following the manufacturer's instructions. Briefly, cells were fixed by incubation with formaldehyde, washed in cold PBS, quenched with glycine, washed, and collected by scraping. Cells then were pelleted, lysed with a Dounce homogenizer and nuclei were isolated. Chromatin was shredded enzymatically and incubated with ChIP-competent antibodies as follows: rabbit polyclonal anti FOXO1 IgG (Santa Cruz Biotechnology) or control polyclonal nonspecific IgG (Cell Signaling, Danvers, MA). Antibodies were precipitated with protein G-coupled beads. Input DNA was also assessed. Triplicate samples were analyzed in a quantitative real-time PCR by using primers and a TaqMan probe (Applied Biosystems) specific for the TNF- α promoter, which has a consensus FOXO1 element. Data are expressed as fold enrichment calculated by $2^{(Ct_{input} - Ct_{ChIP})}$ after quantitative amplification of equivalent amounts of DNA.

Statistical Analysis

Data are presented as mean $\pm$ SEM values. Statistical significance between two groups was established by Student's *t*-test at the $P < 0.05$ level. Analysis of variance with Scheffé's posthoc test was used to assess statistical differences between multiple groups. To establish significance in data represented by immunohistochemical scales, nonparametric analysis was performed with the Kruskal-Wallis test of multiple groups and the Mann-Whitney posthoc test. In the *in vitro* experiments data are expressed as the percent maximum/100 and represents the mean $\pm$ SEM of three independent experiments unless otherwise stated.

Results

Diabetes Up-Regulates Gene Sets Related to Inflammation

To gain insight into how diabetes may affect the fracture repair process, mRNA profiling was performed by using oligonucleotide based microarrays and analyzed by GSEA. The GSEA software examines gene sets that consist of a predefined set of genes that are functionally related in a given pathway.³⁷ Gene sets related to inflam-

Table 1. Gene Set Enrichment Analysis Related to Inflammatory Gene Sets in Fracture Healing

Normal versus diabetic inflammatory gene sets	Number of genes	Day 12				Day 16				Day 22			
		Down-regulated		Up-regulated		Down-regulated		Up-regulated		Down-regulated		Up-regulated	
		<i>P</i>	FDR	<i>P</i>	FDR	<i>P</i>	FDR	<i>P</i>	FDR	<i>P</i>	FDR	<i>P</i>	FDR
Passerini immune	17	<0.01*	0.01*	N/S	N/S	N/S	N/S	<0.01*	0.02*	N/S	N/S	N/S	N/S
Passerini inflammation	21	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.02*	N/S	N/S	N/S	N/S
Inflammatory response pathway	22	N/S	N/S	N/S	N/S	N/S	N/S	0.01*	0.11*	N/S	N/S	N/S	N/S
Inflammatory pathway	29	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
Prostaglandin synthesis regulation	27	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.05*	N/S	N/S	N/S	N/S
Complement pathway	10	<0.01*	0.04*	N/S	N/S	N/S	N/S	<0.01*	0.07*	N/S	N/S	N/S	N/S
Cytokine pathway	13	N/S	N/S	N/S	N/S	N/S	N/S	0.02*	0.10*	N/S	N/S	N/S	N/S
Toll pathway	31	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
Lair pathway	12	<0.01*	0.12*	N/S	N/S	N/S	N/S	<0.01*	0.03*	N/S	N/S	N/S	N/S
Nuclear factor κ B pathway	21	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.09*	N/S	N/S	N/S	N/S
TNF- α nuclear factor κ B dep up	16	<0.01*	0.01*	N/S	N/S	N/S	N/S	<0.01*	0.07*	N/S	N/S	N/S	N/S
TNF pathway	28	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.03*	N/S	N/S	0.02*	0.18*
TNFR1 pathway	23	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.07*	N/S	N/S	N/S	N/S
TNFR2 pathway	17	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.07*	N/S	N/S	N/S	N/S
Stress pathway	25	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
Interleukin-1R pathway	28	N/S	N/S	N/S	N/S	N/S	N/S	0.03*	0.11*	N/S	N/S	N/S	N/S
Interleukin-5 pathway	6	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.10*	N/S	N/S	N/S	N/S
Interleukin-6 pathway	18	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.22*	N/S	N/S	N/S	N/S
Croonquist interleukin-6 stroma up	31	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.10*	N/S	N/S	N/S	N/S
Interleukin-10 pathway	12	N/S	N/S	N/S	N/S	N/S	N/S	0.01*	0.15*	N/S	N/S	N/S	N/S
ST interleukin-13 pathway	6	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.17*	N/S	N/S	N/S	N/S
Interleukin-18 pathway	5	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
NO2/interleukin-12 pathway	15	<0.01*	0.08*	N/S	N/S	N/S	N/S	0.01*	0.16*	N/S	N/S	N/S	N/S
Interleukin-22BP pathway	10	N/S	N/S	N/S	N/S	N/S	N/S	0.01*	0.10*	N/S	N/S	N/S	N/S
CCR5 pathway	18	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
iNOS all up	42	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	0*	0.12*
IFN all up	15	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.09*	N/S	N/S	N/S	N/S
IFN- α up	26	N/S	N/S	N/S	N/S	N/S	N/S	0.01*	0.07*	N/S	N/S	N/S	N/S
IFN- γ up	31	N/S	N/S	N/S	N/S	N/S	N/S	0.01*	0.14*	N/S	N/S	N/S	N/S
IFN- β up	43	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.15*	N/S	N/S	N/S	N/S
CD40 pathway	14	N/S	N/S	N/S	N/S	N/S	N/S	0.03*	0.07*	N/S	N/S	N/S	N/S
PKC pathway	6	N/S	N/S	N/S	N/S	N/S	N/S	0.01*	0.14*	N/S	N/S	N/S	N/S
JNK up	25	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.07*	N/S	N/S	N/S	N/S
P38 MAPK pathway	40	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
Passerini oxidation	16	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.11*	N/S	N/S	N/S	N/S
Cell adhesion molecule activity	84	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.09*	N/S	N/S	N/S	N/S
Monocyte pathway	10	N/S	N/S	N/S	N/S	N/S	N/S	<0.01*	0.05*	N/S	N/S	N/S	N/S
ST granule cell survival pathway	19	0.02*	0.24*	N/S	N/S	N/S	N/S	<0.01*	0.15*	N/S	N/S	N/S	N/S

mRNA profiling was performed on RNA isolated 12, 16, and 22 days after induction of tibial fractures in diabetic and normoglycemic mice. Gene sets related to inflammatory pathways were assessed as described in *Materials and Methods*.

*Significant difference between normoglycemic and diabetic animals with a $P < 0.05$ and a FDR < 0.25 .

N/S, not significant; IFN, interferon; PKC, protein kinase C; NOS, nitrogen dioxide; CCR5, chemokine (C-C motif) receptor 5; iNOS, inducible nitric oxide synthases; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinases.

mation compared diabetic and normoglycemic groups at each time point and were considered significant if the P value was less than 0.05 and a FDR was less than 0.25 for the identified gene set. A total of 38 gene sets related to inflammation were identified (Table 1). The biggest difference between diabetic and normoglycemic groups occurred on day 16 when the diabetic group demonstrated up-regulation of 31 out of 38 pro-inflammatory gene sets ($P < 0.05$, FDR < 0.25). Included were gene sets related to TNF- α and its receptors, interleukins, prostaglandins, complement, interferon and inflammatory cells, among others. The large difference between diabetic and normoglycemic groups noted on day 16 was not observed at an earlier (day 16) or later (day 22) time point. On days 16 and 22 most of the gene sets related to

inflammation were similar in the normoglycemic and diabetic groups having a P -value > 0.05 or a FDR > 0.25 .

Candidate genes that contributed to significant differences between diabetic and normoglycemic fracture healing for the specific gene sets were then identified independently by Student's t -test ($P \leq 0.05$) with a threshold of a 1.5-fold increase or decrease. A large number of up-regulated genes in the diabetic group on day 16 were intercellular mediators and their receptors including cytokines and chemokines (Supplemental Table 2, see <http://ajp.amjpathol.org>). These results point to specific genes that may be particularly important in directly or indirectly signaling pro-inflammatory and pro-osteoclastic events during diabetic fracture healing.

Results obtained with microarrays were compared with those obtained with real-time PCR for selected genes at the 16 day time point when most of the changes were observed (Supplemental Table 3, see <http://ajp.amjpathol.org>). TNF- α , interleukin-1 α , and interleukin-6 mRNA levels were increased 1.7-fold to twofold in the diabetic group compared with normoglycemic as determined by microarray analysis, each of which was significant ($P < 0.05$). When measured by real-time PCR, mRNA values for these genes significantly increased by 1.8-fold to 3.8-fold in the diabetic group compared with the normoglycemic. For real-time PCR, changes for each of the factors were significant ($P < 0.05$).

Diabetes Increases TNF- α Immunopositive Cells in Cartilage during Fracture Healing Which Is Reversed by TNF Inhibition

Because gene sets related to TNF signaling were elevated in the diabetic group, experiments were undertaken to assess the cell types of mesenchymal origin that may express TNF- α in fracture calluses. In addition, the TNF-specific inhibitor, pegsunercept, was applied during fracture healing to determine whether TNF was functionally significant in up-regulating the number of cells that expressed it in diabetic fracture calluses. To accomplish this immunohistochemistry experiments were performed. The following four types of cells were examined by semi-quantitative analysis by using a scale from 0 to 5: proliferative chondrocytes; hypertrophic chondrocytes; and osteoblasts and mesenchymal cells in developing bone and fibroblastic cells in the callus capsule (Figure 1A). Results showed that hypertrophic chondrocytes had the highest level of TNF- α positive cells in all groups ($P < 0.05$) followed by proliferative chondrocytes, osteoblastic and mesenchymal cells in the developing bone, and lastly, fibroblastic cells in the capsule. Diabetes did not alter the number of TNF- α positive cells equally among the cell types examined. The number of capsular fibroblasts or osteoblasts and mesenchymal cells in newly forming bone that expressed TNF- α were similar in normoglycemic and diabetic groups ($P > 0.05$). However, diabetes significantly increased the number of TNF- α positive proliferative and hypertrophic chondrocytes on day 16 ($P < 0.05$). Treatment of the diabetic group with pegsunercept caused a 40% decrease in the TNF- α immunopositive hypertrophic and proliferative chondrocytes ($P < 0.05$). In contrast inhibition of TNF with pegsunercept had no effect on the number of chondrocytes that expressed TNF- α in normoglycemic mice ($P > 0.05$).

To further investigate TNF- α expression specifically by chondrocytes quantitative analysis was performed by measuring the percent hypertrophic and proliferative chondrocytes that were immunopositive. On day 10 there was a 1.4-fold increase in the number of TNF- α immunopositive proliferative and hypertrophic chondrocytes in diabetic mice compared with matched normoglycemic mice ($P < 0.05$; Figure 1, B and C). On day 16 the percent proliferative chondrocytes that expressed TNF- α

Figure 1. Diabetes increases TNF- α expression in chondrocytes in healing fracture calluses, which is reduced by pegsunercept. Femoral fractures were induced in diabetic mice (D), their matched normoglycemic (N), and mice treated with the TNF-specific inhibitor, pegsunercept (PEG) or vehicle alone (PBS) starting on day 10. **A:** The relative expression of TNF- α was examined by immunohistochemistry and assessed from a scale of 0 to 5 as described in *Materials and Methods*. **B:** Quantitative analysis of TNF- α positive cells in the proliferative zone of cartilage. **C:** Quantitative analysis of TNF- α positive cells in hypertrophic chondrocytes. Data are expressed as the mean $\pm$ SEM. * $P < 0.05$ between normal and diabetic groups within the same time point. ** $P < 0.05$ between 16 day diabetic and diabetic pegsunercept treated groups. *** $P < 0.05$ between two time points within a group for a given cell type.

increased 1.5-fold and hypertrophic chondrocytes increased 2.3-fold in the diabetic group ($P < 0.05$; Figure 1, B and C). Treatment of diabetic mice with pegsunercept significantly reduced the percent TNF- α immunopositive proliferative chondrocytes by 48% (Figure 1B) and hypertrophic chondrocytes by 65% (Figure 1C), both of which were significant ($P < 0.05$). In contrast the normoglycemic group showed no change when treated with the TNF inhibitor ($P > 0.05$). A comparison of Figure 1, B and C, indicates that TNF- α immunopositive hypertrophic chondrocytes were threefold to fivefold higher than proliferative chondrocytes in both diabetic and normoglycemic groups ($P < 0.05$; Figure 1, B and C).

TNF Inhibition Reduces a Diabetes Associated Increase in RANKL Positive Chondrocytes

TNF can stimulate bone resorption through RANKL dependent^{41,42} and independent pathways.⁴³ RANKL is a central regulator of bone resorption and has been shown to be up-regulated in diabetic fracture healing.¹⁶ Semi-quantitative analysis was performed (Figure 2A) to establish the cells of mesenchymal origin that express RANKL in diabetic fracture repair and to determine whether their expression was TNF dependent. Results showed that

Figure 2. Diabetes increases RANKL expression in chondrocytes in healing fracture calluses, which is reduced by pegsunercept. **A:** The relative expression of RANKL was examined by immunohistochemistry by using RANKL-specific antibody and assessed from a scale of 0 to 5 as described in *Materials and Methods*. **B:** Quantitative analysis of RANKL positive cells in proliferative chondrocytes. **C:** Quantitative analysis of RANKL positive cells in hypertrophic chondrocytes. Data are expressed as the mean $\pm$ SEM. * $P < 0.05$ between normal and diabetic groups within the same time point. ** $P < 0.05$ between 16 day diabetic and diabetic pegsunercept treated groups.

diabetes caused a significant increase in the percentage of RANKL positive proliferative and hypertrophic chondrocytes ($P < 0.05$). This increase was inhibited by treatment with TNF inhibitor ($P < 0.05$). In contrast, diabetes did not significantly affect the percentage of RANKL immunopositive osteoblastic/mesenchymal or capsule fibroblasts ($P > 0.05$). For both diabetic and normoglycemic groups, the percentage of chondrocytes that expressed RANKL were considerably higher than osteoblasts/mesenchymal cells associated with newly formed bone and fibroblasts in the capsule.

Quantitative analysis was then performed to more precisely define the expression of RANKL in chondrocytes (Figure 2, B and C). Diabetes caused a twofold increase in proliferative chondrocytes that were RANKL positive on day 16, which was reduced by more than 50% by treatment with pegsunercept ($P < 0.05$; Figure 2B). RANKL positive hypertrophic chondrocytes were increased approximately twofold by diabetes on days 10 and 16. Inhibition of TNF reduced the number of chondrocytes that expressed RANKL to normal levels ($P < 0.05$; Figure 2, B and C). Results described above indicate that diabetes enhances the expression of TNF- α and RANKL in diabetic fracture calluses in chondrocytes and that their expression is largely reduced by TNF inhibition.

Figure 3. TNF inhibition reverses the effect of diabetes on osteoclast numbers, cartilage resorption, and callus area. Femoral fractures were induced as described in Figure 1. **A:** Osteoclasts were counted in sections as bone or cartilage lining multinucleated tartrate-resistant acid phosphatase positive cells and expressed as the number per mm² cartilage and bone area in each callus. **B:** Transverse-sections were stained with safranin-O/fast green stain to measure cartilage area. **C:** Sections were stained with H&E to measure callus area. Data are expressed as mean $\pm$ SEM. * $P < 0.05$ between normal and diabetic groups within the same time point. ** $P < 0.05$ between 16 day diabetic and pegsunercept treated diabetic mice. *** $P < 0.05$ between the two time points within the group.

TNF Inhibition Decreases Diabetes Enhanced Cartilage Degradation and Osteoclastogenesis

To determine whether increased TNF- α represented a mechanism for diabetes-enhanced loss of cartilage that we previously reported,¹⁶ mice were treated with pegsunercept and the number of osteoclasts and changes in cartilage were measured. On day 10 the number of osteoclasts was small and similar in the diabetic and normoglycemic fracture calluses (Figure 3A). On day 16 there was a significant increase in the number of osteoclasts in both groups. However, the number in the diabetic group was almost fourfold higher than the normoglycemic group ($P < 0.05$; Figure 3A). Inhibition of TNF reduced the number of osteoclasts in the diabetic group by 58% but had no effect on the normoglycemic mice ($P < 0.05$) (Supplemental Figure 1, see <http://ajp.amjpathol.org>). The impact of osteoclast resorption of mineralized cartilage was assessed by measuring the cartilage area in each fracture callus. There was no difference in the amount of cartilage in the normal and diabetic groups at an early time point, day 10 (Figure 3B). On day 16 the amount of cartilage in the diabetic group

was 42% less than the normoglycemic group ($P < 0.05$; Figure 3B). This decrease was reversed by pegsunercept treatment so that amount of cartilage was similar in the normoglycemic and diabetic group treated when TNF was inhibited ($P > 0.05$; Figure 3B) (Supplemental Figure 2, see <http://ajp.amjpathol.org>). Inhibition of TNF did not affect the cartilage area in the normoglycemic mice consistent with the lack of effect on the number of TNF- α and RANKL positive chondrocytes and the number of osteoclasts in the normal control mice.

Because the amount of cartilage present impacts the size of the callus, callus area was measured in each section. The callus size was significantly smaller in the diabetic group on day 16 and the decrease was reversed by treatment with pegsunercept ($P < 0.05$; Figure 3C). The TNF blocker did not affect callus size in the normoglycemic group ($P > 0.05$).

TNF Stimulation Increases FOXO1 Activity and Up-Regulates FOXO1 and TNF- α mRNA in Cells with Chondrocytic Phenotype

The role of TNF in the expression of pro-osteoclastogenic factors in chondrocytes was further studied *in vitro* by using C3H10T1/2 murine mesenchymal stem cell line and ATDC5 chondrogenic cells that can differentiate into chondrocytes.^{40,41,44,45} Establishment of the hypertrophic chondrocytic phenotype under BMP-2 treatment was demonstrated by a fivefold and 14-fold increase in type-II and type-X collagen mRNA levels following induction with BMP-2 in ATDC5 cells and a threefold and 15-fold increase in type-II and type-X collagen mRNA levels in BMP-2 treated C3H10T1/2 cells ($P < 0.05$; see Supplemental Table 4 at <http://ajp.amjpathol.org>). Because we have recently found that FOXO1 mediates TNF induced expression of pro-apoptotic genes,²⁸ we determined whether TNF induced expression of pro-osteoclastogenic factors by chondrocytic cells was FOXO1 dependent by using RNA interference.

To investigate FOXO1 in cells with a hypertrophic chondrocyte phenotype C3H10T1/2 cells were first induced with BMP-2 and then stimulated with TNF- α . FOXO1 DNA binding was increased by TNF- α up to twofold in a dose-dependent manner ($P < 0.05$; Figure 4A) and TNF- α stimulated almost a threefold increase in FOXO1 mRNA levels ($P < 0.05$; Figure 4B). In contrast, undifferentiated C3H10T1/2 mesenchymal cells did not exhibit a change in FOXO1 DNA binding or in FOXO1 mRNA or TNF- α mRNA levels when stimulated with TNF- α (Supplemental Figure 3, see <http://ajp.amjpathol.org>). The potential role of FOXO1 in mediating TNF- α induced mRNA in chondrogenic cells was examined by siRNA. BMP-2 treated C3H10T1/2 cells were transfected with FOXO1 siRNA or scrambled siRNA followed by TNF stimulation (20 ng/ml). FOXO1 siRNA significantly knocked down the mRNA levels of FOXO1 by over 82% ($P < 0.05$; Figure 4B), whereas scrambled siRNA had no effect ($P > 0.05$). A fivefold increase in TNF- α mRNA levels was shown in TNF stimulated chondrogenic C3H10T1/2 cells ($P < 0.05$; Figure 5A). Knockdown of FOXO1 significantly

Figure 4. TNF- α stimulates FOXO1 activity and FOXO1 mRNA levels in C3H10T1/2 cells with a chondrogenic phenotype. C3H10T1/2 cells were treated with BMP-2. **A:** Cells were then stimulated with TNF- α (0 to 20 ng/ml) as described in *Materials and Methods*. Nuclear proteins were assessed for FOXO1 DNA binding activity by transcription factor ELISA. **B:** Cells were stimulated with TNF- α (20 ng/ml) and total RNA was extracted and RT-PCR was performed to measure FOXO1 and mRNA levels. Data are expressed as the percent maximum/100 and represent the mean $\pm$ SEM of three independent experiments. * $P < 0.05$ in TNF- α -stimulated cells compared with cells without TNF- α . ** $P < 0.05$ between FOXO1 siRNA and scrambled siRNA groups.

reduced TNF mRNA levels by approximately 85% in these cells ($P < 0.05$; Figure 5A). In contrast scrambled siRNA had no effect ($P > 0.05$). Similar experiments were performed in ATDC5 cells and results showed that TNF- α stimulation increased FOXO1 DNA binding activity, FOXO1 and TNF- α mRNA levels in ATDC5 cells with or without BMP-2 pretreatment, all of which were reduced by transfection with FOXO1 siRNA (data not shown).

To investigate TNF- α and the possible regulation by FOXO1 in chondrocytes in more depth, ATDC5 cells were pretreated with BMP-2 for 6 days then stimulated with TNF- α (20 ng/ml) for 30 or 60 minutes and compared with unstimulated before analysis of FOXO1/TNF- α promoter association by ChIP. TNF- α stimulation resulted in significant enrichment of FOXO1 association with the TNF- α promoter compared with control IgG precipitates (Figure 5B) in two independent experiments.

FOXO1 Knockdown Decreases TNF Stimulated Up-Regulation of Proinflammatory and Pro-osteoclastogenic Factors in Chondrocytes

Several other pro-osteoclastogenic factors were examined, including RANKL, M-CSF, interleukin-1 α and interleukin-6 in ATDC5 and C3H10T1/2 cells with a BMP-2-induced hypertrophic chondrocyte phenotype (Figure 6). TNF stimulated an eightfold and 5.8-fold increase in RANKL mRNA levels, respectively ($P < 0.05$, Figure 6, A and B). FOXO1 siRNA caused a 69% reduction in RANKL

Figure 5. A: TNF- α mRNA levels are increased by TNF- α stimulation and decreased by FOXO1 knockdown in C3H10T1/2 cells with a chondrogenic phenotype. C3H10T1/2 cells were treated with BMP-2. A group of cells were then stimulated with TNF- α (20 ng/ml), RNA extracted and RT-PCR was performed to measure TNF- α mRNA levels. Data are expressed as the percent maximum/100 and represent the mean $\pm$ SEM of three independent experiments. * P < 0.05 in TNF- α -stimulated cells compared with cells without TNF- α . ** P < 0.05 between FOXO1 siRNA and scrambled siRNA groups. **B:** TNF- α stimulates FOXO1 association with the TNF- α promoter as measured by ChIP. ATDC5 cells were treated with BMP-2 for 6 days and then were either left untreated or stimulated with TNF- α for 30 or 60 minutes. Chromatin fragments were immunoprecipitated with anti-FOXO1 antibody and quantified by real-time PCR. All ChIP values are normalized to PCR signal from samples precipitated with a control nonspecific IgG, whose value was set to 1. Data are from experiment with triplicate samples and representative of two independent experiments.

mRNA level in TNF- α stimulated ATDC5 and a 78% decrease in C3H10T1/2 cells with a chondrogenic phenotype (P < 0.05; Figure 6, A and B), whereas transfection with scrambled siRNA had no effect (P > 0.05). TNF- α stimulated an eight fold increase in the mRNA levels of M-CSF in ATDC5 and a 4.5-fold increase in C3H10T1/2 cells that had been induced with BMP-2. FOXO1 knockdown decreased M-CSF gene expression by 65 to 75% in these cells (P < 0.05; Figure 6, C and D). Transfection with scrambled siRNA had no effect (P > 0.05). TNF- α increased interleukin-1 α mRNA levels by 23-fold in BMP-2-induced ATDC5 cells and 12.5-fold in BMP-2-induced C3H10T1/2 cells (P < 0.05; Figure 6, E and F). Silencing FOXO1 reduced interleukin-1 α mRNA levels by 58% in ATDC5 cells and by 90% in C3H10T1/2 cells with a chondrogenic phenotype compared with scrambled siRNA (P < 0.05). Interleukin-6 mRNA levels were enhanced by 90-fold in BMP-2-induced ATDC5 cells on TNF- α stimulation (P < 0.05; Figure 6G). FOXO1 knockdown decreased interleukin-6 mRNA levels in TNF- α stimulated cells by 84% (P < 0.05; Figure 6G). In C3H10T1/2 cells with hypertrophic chondrocyte phenotype, interleukin-6 mRNA levels were 50-fold higher in

Figure 6. FOXO1 RNA interference inhibits TNF- α stimulated up-regulation of pro-inflammatory and pro-osteoclastogenic genes in differentiated chondrocytes. Chondrocytes were differentiated with (200 ng/ml) BMP-2 for 6 days in ATDC5 cells (**A**, **C**, **E**, and **G**) and with (100 ng/ml) BMP-2 for 4 days in C3H10T1/2 cells (**B**, **D**, **F**, and **H**). In some groups, cells were transfected with FOXO1 siRNA or scrambled siRNA and/or stimulated with TNF- α (20 ng/ml) for 6 hours as indicated. Total RNA was collected and RT-PCR was performed to examine RANKL, M-CSF, interleukin-1 α and interleukin-6 mRNA levels. Data are expressed as the percent maximum/100 and represents the mean $\pm$ SEM of three independent experiments. * P < 0.05 in TNF- α stimulated cells compared with cells without TNF- α . ** P < 0.05 between FOXO1 siRNA and scrambled siRNA groups.

TNF stimulated cells (P < 0.05; Figure 6H). Transfection of FOXO1 siRNA reduced interleukin-6 mRNA levels by over 90% (P < 0.05). Collectively, the results showed that TNF- α stimulated at least a fourfold increase in mRNA levels of each pro-osteoclastogenic gene and knockdown of FOXO1 with FOXO1 siRNA caused a significant 50 to 90% reduction in the mRNA level in ATDC5 cells or C3H10T1/2 cells with a hypertrophic chondrocytic phenotype (P < 0.05; Figure 6).

Diabetes Enhances FOXO1 DNA Binding Activity and Nuclear Translocation

The *in vitro* studies above indicate that TNF- α stimulates expression of several pro-osteoclastogenic genes, particularly those with a chondrocytic phenotype. This is consistent with *in vivo* results demonstrating that high levels of TNF- α and RANKL expression were found in hypertrophic chondrocytes, particularly in diabetic fracture calluses, in a TNF-dependent manner. We determined whether FOXO1 was affected by diabetes. No

Figure 7. Diabetes increases FOXO1 DNA binding activity and FOXO1 nuclear translocation in healing fractures. **A:** Nuclear protein was extracted from femoral fracture calluses and FOXO1 DNA binding assay was measured by transcription factor ELISA. The data represent the mean of the three separate experiments $\pm$ SEM. **B and C:** Transverse sections from calluses were incubated with anti-FOXO1 IgG or matched control IgG and nuclear translocation was assessed by confocal scanning laser microscopy by counting the percent proliferative (**B**) and hypertrophic (**C**) chondrocytes with unambiguous FOXO1 nuclear translocation. Data are expressed as the mean $\pm$ SEM. * $P < 0.05$ between normal and diabetic mice within the same time point. ** $P < 0.05$ between 16 day diabetic and diabetic pegsunercept (PEG) treated mice. *** $P < 0.05$ between days 10 and day 16 within a given group.

difference was noted in FOXO1 DNA binding activity in nuclear proteins extracted from diabetic and normoglycemic fracture calluses on day 10 ($P > 0.05$; Figure 7A). However, on day 16 the diabetic fracture calluses had 70% more FOXO1 DNA binding activity than matched normoglycemic fracture calluses ($P < 0.05$; Figure 7A). When FOXO1 is activated it is translocated to the nucleus and on deactivation is quickly transported out of the nucleus.^{46,47} We examined FOXO1 nuclear translocation that occurred in chondrocytes in diabetic fractures by confocal laser scanning microscopy. On day 16, diabetes caused a 2.9-fold increase in the percent proliferative and a 3.1-fold increase in the percent hypertrophic chondrocytes with FOXO1 nuclear translocation compared with matched normoglycemic controls ($P < 0.05$; Figure 7, B and C). Inhibition of TNF in the diabetic group resulted in a 55% decrease in the percent proliferative chondrocytes and a 62% decrease in the percent hypertrophic chondrocytes that had FOXO1 nuclear translocation ($P < 0.05$). In contrast, TNF inhibition had no significant effect on FOXO1 nuclear translocation in normoglycemic mice ($P > 0.05$). From day 10 to day 16 there was 2.9-fold increase in proliferative chondrocytes and a 5.5-fold increase in hypertrophic chondrocytes with FOXO1 nuclear translocation in the diabetic

group ($P < 0.05$). There were no differences in FOXO1 nuclear translocation between diabetic mice and normoglycemic mice at day 10 ($P > 0.05$). A comparison of Figure 7, B and C, indicates that on day 16 the percent positive chondrocytes with FOXO1 nuclear translocation in hypertrophic chondrocytes was 2.8-fold higher than the proliferative chondrocytes in the diabetic fracture specimens ($P < 0.05$).

Discussion

We previously reported that diabetic fracture healing was characterized by the accelerated loss of cartilage.¹⁶ To examine mechanisms by which this may occur, mRNA profiling and GSEA were performed by focusing on time points associated with the cartilage degradation. Several inflammatory pathways were found to be up-regulated by diabetes. This is potentially important because many of these pathways are linked to stimulation of osteoclast formation either directly or indirectly through TNF- α .⁴⁸ Interestingly, this up-regulation was prominent on day 16 but was relatively absent on days 12 and 22. We previously reported that pro-osteoclastogenic factors were up-regulated in two phases in normal fracture repair with the first occurring within a few days of fracture and that latter occurring between 14 to 21 days.^{49,50} Thus, diabetes exerted an effect when resorption of cartilage is particularly high. This result indicates that diabetes causes a global up-regulation of genes that regulate pro-inflammatory pathways many of which are linked to osteoclastogenesis.

Two important pro-osteoclastogenic factors are TNF- α and RANKL. During fracture repair, inhibition of RANKL greatly reduces cartilage resorption during the transition from cartilage to bone and leads to a substantial increase in new bone formation that is associated with a delay in cartilage removal.⁵¹ When examined by immunohistochemistry the percent TNF- α and RANKL positive chondrocytes was significantly higher in proliferative and hypertrophic chondrocytes compared with mesenchymal and osteoblastic cells associated with newly forming bone and with fibroblastic cells associated with the fibrous capsule. In addition TNF- α and RANKL expressing chondrocytes were significantly higher in the diabetic mice compared with normoglycemic mice. When TNF- α was inhibited the percent hypertrophic and proliferative chondrocytes that express TNF- α and RANKL were both reduced in the diabetic group but were not affected by the TNF blocker in the normoglycemic fracture specimens. The expression by mesenchymal and osteoblastic cells in forming bone and fibroblasts in the capsule were not affected by TNF inhibition. This would suggest that the high level expression of pro-osteoclastogenic factors in chondrocytes is mediated at least in part by TNF dysregulation in diabetic fracture healing. The link between enhanced TNF- α and diabetes-enhanced osteoclastogenesis was further supported by significantly reduced osteoclast numbers and diminished cartilage loss in diabetic mice treated with pegsunercept in diabetic fracture healing. In contrast, TNF inhibition had little effect in normoglycemic mice. These findings indicate that

TNF- α is particularly important in enhancing osteoclastogenesis in diabetic mice. In mice with genetic ablation of TNF receptors there is a subtle delay in the removal of mineralized cartilage.⁴⁹ This result differs somewhat from the data obtained here in normoglycemic animals. The difference may be due to the fact that TNF was inhibited starting on day 10 after fracture in experiments, which may have a different impact compared with mice with congenitally absent TNF receptor signaling due to genetic modification. Nevertheless, it is apparent that TNF plays a more prominent role in osteoclastogenesis in diabetic mice than in normoglycemic animals.

We have previously reported that the transcription factor FOXO1 mediates pro-apoptotic gene expression stimulated by TNF- α .²⁸ Because TNF dysregulation appeared to play a significant role in diabetes-enhanced expression of resorptive factors, experiments were undertaken to examine the role of FOXO1 in TNF- α induced pro-osteoclastogenic genes in chondrocytic cells *in vitro*. The results demonstrated that TNF- α significantly increased FOXO1 mRNA levels and DNA binding activity in cells with a chondrogenic phenotype but not in immature C3H10T1/2 mesenchymal cells, indicating the acquisition of the chondrogenic phenotype rendered the cells susceptible to TNF-induced FOXO1. FOXO1 knockdown significantly reduced the capacity of TNF- α to induce mRNA levels of TNF- α , RANKL, M-CSF, interleukin-1- α , and interleukin-6 in chondrogenic C3H10T1/2 cells and ATDC5 cells.

In vivo results indicate that FOXO1 DNA binding activity is significantly elevated in diabetic fracture calluses on day 16 when pro-inflammatory gene sets were up-regulated. FOXO1 nuclear translocation, particularly in hypertrophic chondrocytes was significantly higher in diabetic fracture calluses. Inhibition of TNF- α by pegsunercept treatment reduced translocation of FOXO1 to the nucleus demonstrating that high levels of TNF- α contributed to elevated FOXO1 activation. Chromatin immunoprecipitation experiments demonstrate an association between FOXO1 and the TNF- α promoter in chondrocytes stimulated with TNF- α , further establishing a relationship between FOXO1 and pro-resorptive events in these cells. Because inflammatory mediators work in networks a number of inflammatory cytokines, reactive oxygen species, or other mediators may be affected secondary to TNF inhibition. Thus, diabetes-enhanced TNF- α may also stimulate FOXO1 indirectly through the induction of other pro-inflammatory factors.

The results reported in this study suggested that TNF- α dysregulation plays a prominent role in the recently identified catabolic events associated with diabetic fracture healing.¹⁶ When TNF was blocked the loss of cartilage was reduced, osteoclast numbers were decreased, and the expression of pro-osteoclastogenic factors RANKL and TNF- α by chondrocytes was diminished. Furthermore, TNF may exert its effect through activation of the transcription factor FOXO1 based on evidence that FOXO1 is elevated in chondrocytes of diabetic fracture calluses in a TNF-dependent manner and that FOXO1 knockdown significantly reduces TNF- α -induced expression of pro-osteoclastogenic factors in chondrocytic cells

in vitro. This suggests a potential mechanism by which diabetes-enhanced TNF- α may induce FOXO1 activation, which in turn cause stimulation of inflammatory and resorptive processes leading to greater loss of cartilage in diabetic fractures. Others have proposed that FOXO may be induced in bone by reactive oxygen species and exert an effect by antagonizing Wnt signaling, an essential stimulus for osteoblastogenesis.^{52,53} In this regard it is interesting to note the obligate requirement of TNF signaling in cultured articular chondrocytes for inducible nitric oxide synthase synthesis.⁵⁴ Thus, FOXO1 may have an effect on mineralized tissue that involves osteoclastogenesis in addition to its putative effect on osteoblastic cells through the Wnt pathway.

References

1. Tuominen JT, Impivaara O, Puukka P, Ronnema T: Bone mineral density in patients with type 1 and type 2 diabetes. *Diabetes Care* 1999, 22:1196–1200
2. Jehle PM, Jehle DR, Mohan S, Bohm BO: Serum levels of insulin-like growth factor system components and relationship to bone metabolism in Type 1 and Type 2 diabetes mellitus patients. *J Endocrinol* 1998, 159:297–306
3. Vestergaard P, Rejnmark L, Mosekilde L: Relative fracture risk in patients with diabetes mellitus, and the impact of insulin and oral antidiabetic medication on relative fracture risk. *Diabetologia* 2005, 48:1292–1299
4. Leger J, Marinovic D, Alberti C, Dorgeret S, Chevenne D, Marchal CL, Tubiana-Rufi N, Sebag G, Czernichow P: Lower bone mineral content in children with type 1 diabetes mellitus is linked to female sex, low insulin-like growth factor type I levels, and high insulin requirement. *J Clin Endocrinol Metab* 2006, 91:3947–3953
5. Kemink SA, Hermus AR, Swinkels LM, Lutterman JA, Smals AG: Osteopenia in insulin-dependent diabetes mellitus: prevalence and aspects of pathophysiology. *J Endocrinol Invest* 2000, 23:295–303
6. Davidson TI, Bodey WN: Factors influencing survival following fractures of the upper end of the femur. *Injury* 1986, 17:12–14
7. Ivers RQ, Cumming RG, Mitchell P, Peduto AJ: Diabetes and risk of fracture: the Blue Mountains Eye Study. *Diabetes Care* 2001, 24:1198–1203
8. Nicodemus KK, Folsom AR: Type 1 and type 2 diabetes and incident hip fractures in postmenopausal women. *Diabetes Care* 2001, 24:1192–1197
9. Cozen L: Does diabetes delay fracture healing? Does diabetes delay fracture healing? *Clin Orthop* 1972, 82:134–140
10. Loder R: The influence of diabetes mellitus on the healing of closed fractures. *Clin Orthop* 1988, 232:210–216
11. Herskind AM, Christensen K, Norgaard-Andersen K, Andersen JF: Diabetes mellitus and healing of closed fractures. *Diabetes Metab* 1992, 18:63–64
12. Herbsman H, Powers J, Hirschman A, Shaftan G: Retardation of fracture healing in experimental diabetes. *J Surg Res* 1968, 8:424–431
13. Gooch HL, Hale JE, Fujioka H, Balian G, Hurwitz SR: Alterations of cartilage and collagen expression during fracture healing in experimental diabetes. *Connect Tissue Res* 2000, 41:81–91
14. Funk JR, Hale JE, Carmines D, Gooch HL, Hurwitz SR: Biomechanical evaluation of early fracture healing in normal and diabetic rats. *J Orthop Res* 2000, 18:126–132
15. Gerstenfeld L, Cullinan D, Barnes G, Graves D, Einhorn T: Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *J Cell Biochem* 2003, 88:873–884
16. Kayal RA, Tsatsas D, Bauer MA, Allen B, Al-Sebaei MO, Kakar S, Leone CW, Morgan EF, Gerstenfeld LC, Einhorn TA, Graves DT: Diminished bone formation during diabetic fracture healing is related to the premature resorption of cartilage associated with increased osteoclast activity. *J Bone Miner Res* 2007, 22:560–568

17. Chambers TJ, Revell PA, Fuller K, Athanasou NA: Resorption of bone by isolated rabbit osteoclasts. *J Cell Sci* 1984, 66:383-399
18. Krakauer J, McKenna M, Burdener N, Rao D, Whitehouse F, Pafitt A: Bone loss and bone turnover in diabetes. *Diabetes* 1995, 44:775-782
19. Suzuki K, Kurose T, Takizawa M, Maruyama M, Ushikawa K, Kikuyama M, Sugimoto C, Seino Y, Nagamatsu S, Ishida H: Osteoclastic function is accelerated in male patients with type 2 diabetes mellitus: the preventive role of osteoclastogenesis inhibitory factor/osteoprotegerin (OCIF/OPG) on the decrease of bone mineral density. *Diabetes Res Clin Pract* 2005, 68:117-125
20. Gough A, Abraha H, Li F, Purewal TS, Foster AV, Watkins PJ, Moniz C, Edmonds ME: Measurement of markers of osteoclast and osteoblast activity in patients with acute and chronic diabetic Charcot neuroarthropathy. *Diabet Med* 1997, 14:527-531
21. Jirkovska A, Kasalicky P, Boucek P, Hosova J, Skibova J: Calcaneal ultrasonometry in patients with Charcot osteoarthropathy and its relationship with densitometry in the lumbar spine and femoral neck and with markers of bone turnover. *Diabet Med* 2001, 18:495-500
22. Liu R, Bai HS, Desta T, Krothapalli N, Alyassi M, Luan Q, Graves D: Diabetes enhances periodontal bone loss through enhanced resorption and diminished bone formation. *J Dent Res* 2006, 85:510-514
23. Bjorgaas M, Haug E, Johnsen H: The urinary excretion of deoxypyridinium cross-links is higher in diabetic than in nondiabetic adolescents. *Calcif Tissue Int* 1999, 65:121-124
24. Naqvi A, Cuchacovich R, Saketkoo L, Espinoza LR: Acute charcot arthropathy successfully treated with pamidronate: long-term follow-up. *Am J Med Sci* 2008, 335:145-148
25. Goova MT, Li J, Kislinger T, Qu W, Lu Y, Bucciarelli LG, Nowygrod S, Wolf BM, Caliste X, Yan SF, Stern DM, Schmidt AM: Blockade of receptor for advanced glycation end-products restores effective wound healing in diabetic mice. *Am J Pathol* 2001, 159:513-525
26. Engebretson S, Chertog R, Nichols A, Hey-Hadavi J, Celenti R, Grbic J: Plasma levels of tumor necrosis factor-alpha in patients with chronic periodontitis and type 2 diabetes. *J Clin Periodontol* 2007, 34:18-24
27. Pessara U, Koch N: Tumor necrosis factor alpha regulates expression of the major histocompatibility complex class II-associated invariant chain by binding of an NF-kappa B-like factor to a promoter element. *Mol Cell Biol* 1990, 10:4146-4154
28. Alikhani M, Alikhani Z, Graves DT: FOXO1 functions as a master switch that regulates gene expression necessary for tumor necrosis factor-induced fibroblast apoptosis. *J Biol Chem* 2005, 280:12096-12102
29. Accili D, Arden KC: FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell* 2004, 117:421-426
30. Greer EL, Brunet A: FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene* 2005, 24:7410-7425
31. Rokudai S, Fujita N, Kitahara O, Nakamura Y, Tsuruo T: Involvement of FKHR-dependent TRADD expression in chemotherapeutic drug-induced apoptosis. *Mol Cell Biol* 2002, 22:8695-8708
32. Gilley J, Coffey PJ, Ham J: FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. *J Cell Biol* 2003, 162:613-622
33. Kayal RA, Alblowi J, McKenzie E, Krothapalli N, Silkman L, Gerstenfeld L, Einhorn TA, Graves DT: Diabetes causes the accelerated loss of cartilage during fracture repair which is reversed by insulin treatment. *Bone* 2009, 44:357-363
34. Gerstenfeld LC, Wronski TJ, Hollinger JO, Einhorn TA: Application of histomorphometric methods to the study of bone repair. *J Bone Miner Res* 2005, 20:1715-1722
35. Gerstenfeld LC, Alkhiary YM, Krall EA, Nicholls FH, Stapleton SN, Fitch JL, Bauer M, Kayal R, Graves DT, Jepsen KJ, Einhorn TA: Three-dimensional reconstruction of fracture callus morphogenesis. *J Histochem Cytochem* 2006, 54:1215-1228
36. Wang K, Vishwanath P, Eichler GS, Al-Sebaei MO, Edgar CM, Einhorn TA, Smith TF, Gerstenfeld LC: Analysis of fracture healing by large-scale transcriptional profile identified temporal relationships between metalloproteinase and ADAMTS mRNA expression. *Matrix Biol* 2006, 25:271-281
37. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP: Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 2005, 102:15545-15550
38. He H, Liu R, Desta T, Leone C, Gerstenfeld L, Graves D: Diabetes causes decreased osteoclastogenesis, reduced bone formation, and enhanced apoptosis of osteoblastic cells in bacteria stimulated bone loss. *Endocrinology* 2004, 145:447-452
39. Shea CM, Edgar CM, Einhorn TA, Gerstenfeld LC: BMP treatment of C3H10T1/2 mesenchymal stem cells induces both chondrogenesis and osteogenesis. *J Cell Biochem* 2003, 90:1112-1127
40. Denker AE, Haas AR, Nicoll SB, Tuan RS: Chondrogenic differentiation of murine C3H10T1/2 multipotential mesenchymal cells: i. Stimulation by bone morphogenetic protein-2 in high-density micromass cultures. *Differentiation* 1999, 64:67-76
41. Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL: TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J Clin Invest* 2000, 106:1481-1488
42. Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S: Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. *Bone* 1999, 25:255-259
43. Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, Nakagawa N, Kinoshita M, Yamaguchi K, Shima N, Yasuda H, Morinaga T, Higashio K, Martin TJ, Suda T: Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. *J Exp Med* 2000, 191:275-286
44. Fischer L, Boland G, Tuan R: Wnt signaling during BMP-2 stimulation of mesenchymal chondrogenesis. *J Cell Biochem* 2002, 84:816-831
45. Shukunami C, Ohta Y, Sakuda M, Hiraki Y: Sequential progression of the differentiation program by bone morphogenetic protein-2 in chondrogenic cell line ATDC5. *Exp Cell Res* 1998, 241:1-11
46. Birkenkamp KU, Coffey PJ: Regulation of cell survival and proliferation by the FOXO (Forkhead box, class O) subfamily of Forkhead transcription factors. *Biochem Soc Trans* 2003, 31:292-297
47. Datta SR, Brunet A, Greenberg ME: Cellular survival: a play in three acts. *Genes Dev* 1999, 13:2905-2927
48. Teitelbaum SL: Osteoclasts: What do they do and how do they do it? *Am J Pathol* 2007, 170:427-435
49. Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Tsay A, Fitch J, Barnes GL, Graves DT, Einhorn TA: Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption. *J Bone Miner Res* 2003, 18:1584-1592
50. Kon T, Cho TJ, Aizawa T, Yamazaki M, Nooh N, Graves D, Gerstenfeld LC, Einhorn TA: Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. *J Bone Miner Res* 2001, 16:1004-1014
51. Gerstenfeld L, Sacks D, Pelis M, Mason Z, Graves D, Barrero M, Ominsky M, Kostenuik P, Morgan E, Einhorn T: Comparison of effects of the bisphosphonate alendronate versus the RANKL inhibitor denosumab on murine fracture healing. *O J Bone Miner Res* 2008, 24:196-208
52. Essers MA, Weijzen S, de Vries-Smits AM, Saarloos I, de Ruiter ND, Bos JL, Burgering BM: FOXO transcription factor activation by oxidative stress mediated by the small GTPase Rac and JNK. *EMBO J* 2004, 23:4802-4812
53. Almeida M, Han L, Martin-Millan M, O'Brien CA, Manolagas SC: Oxidative stress antagonizes Wnt signaling in osteoblast precursors by diverting beta-catenin from T cell factor- to forkhead box O-mediated transcription. *J Biol Chem* 2007, 282:27298-27305
54. Cho TJ, Lehmann W, Edgar C, Sadeghi C, Hou A, Einhorn TA, Gerstenfeld LC: Tumor necrosis factor alpha activation of the apoptotic cascade in murine articular chondrocytes is associated with the induction of metalloproteinases and specific pro-resorptive factors. *Arthritis Rheum* 2003, 48:2845-2854