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Abstract

Chemokines are thought to play an important role in several aspects of bone metabolism including the recruitment of leukocytes and the formation of osteoclasts. We investigated the impact of diabetes on chemokine expression in normal and diabetic fracture healing. Fracture of the femur was performed in streptozotocin-induced diabetic and matched normoglycemic control mice. Microarray analysis was carried out and chemokine mRNA levels *in vivo* were assessed. CCL4 were examined in fracture calluses by immunohistochemistry and the role of TNF in diabetes-enhanced expression was investigated by treatment of animals with the TNF-specific inhibitor, pegsunercept. *In vitro* studies were conducted with ATDC5 chondrocytes. Diabetes significantly upregulated mRNA levels of several chemokines *in vivo* including CCL4, CCL8, CCL6, CCL11, CCL20, CCL24, CXCL2, CXCL5 and chemokine receptors CCR5 and CXCR4. Chondrocytes were identified as a significant source of CCL4 and its expression in diabetic fractures was dependent on TNF ($P < 0.05$). TNF- α significantly increased mRNA levels of several chemokines *in vitro* which were knocked down with FOXO1 siRNA ($P < 0.05$). CCL4 expression at the mRNA and proteins levels was induced by FOXO1 over-expression and reduced by FOXO1 knockdown. The current studies point to the importance of TNF- α as a mechanism for diabetes enhanced chemokine expression by chondrocytes, which may contribute to the accelerated loss of cartilage observed in diabetic fracture healing. Moreover, *in vitro* results point to FOXO1 as a potentially important transcription factor in mediating this effect.

Keywords

Animals, Chemokines, Chondrocytes, Diabetes Mellitus, Experimental, Fracture Healing, Immunohistochemistry, Mice, RNA Interference, RNA, Messenger, Up-Regulation

Disciplines

Amino Acids, Peptides, and Proteins | Dentistry | Endocrinology, Diabetes, and Metabolism | Osteopathic Medicine and Osteopathy

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Chemokine Expression is Upregulated in Chondrocytes in Diabetic Fracture Healing

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Abstract

Chemokines are thought to play an important role in several aspects of bone metabolism including the recruitment of leukocytes and the formation of osteoclasts. We investigated the impact of diabetes on chemokine expression in normal and diabetic fracture healing. Fracture of the femur was performed in streptozotocin-induced diabetic and matched normoglycemic control mice. Microarray analysis was carried out and chemokine mRNA levels *in vivo* were assessed. CCL4 were examined in fracture calluses by immunohistochemistry and the role of TNF in diabetes-enhanced expression was investigated by treatment of animals with the TNF-specific inhibitor, pegsunercept. *In vitro* studies were conducted with ATDC5 chondrocytes. Diabetes significantly upregulated mRNA levels of several chemokines *in vivo* including CCL4, CCL8, CCL6, CCL11, CCL20, CCL24, CXCL2, CXCL5 and chemokine receptors CCR5 and CXCR4. Chondrocytes were identified as a significant source of CCL4 and its expression in diabetic fractures was dependent on TNF in diabetic fractures ($P < 0.05$). TNF- significantly increased mRNA levels of several chemokines *in vitro* which were knocked down with FOXO1 siRNA ($P < 0.05$). CCL4 expression at the mRNA and proteins levels was induced by FOXO1 over-expression and reduced by FOXO1 knockdown. The current studies point to the importance of TNF- as a mechanism for diabetes enhanced chemokine expression by chondrocytes, which may contribute to the accelerated loss of cartilage observed in diabetic fracture healing. Moreover, *in vitro* results point to FOXO1 as a potentially important transcription factor in mediating this effect.

Introduction

Chemokines are small (8–11 kDa) chemotactic cytokines secreted by many cell types in response to growth factors, inflammatory cytokines, and cancer cells [1]. Chemokines are classified into two major subfamilies by their N terminal cysteines, CXC, CC, and two

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minor families, C and CX3C. Some chemokines interact with a single high affinity chemokine receptor while others bind multiple chemokine receptors [1].

Osteoclasts originate from hematopoietic precursors of the monocyte-macrophage lineage that reside within the bone marrow. Chemokines that are chemotactic for cells of this lineage are thought to be important in trafficking of osteoclast precursors and to modulate the lifespan of osteoclasts [2, 3]. A number of chemokines have been reported to recruit osteoclast precursors or stimulate osteoclastogenesis including CCL2, CCL3, CCL4, CXCL8 and CXCL12 [4]. In conditions where there is increased bone resorption these chemokines are elevated such as arthritis, osteolytic bone disease of multiple myeloma and periodontal disease [5-8]. CCL3 and CCL4 are constitutively secreted by multiple myeloma cells and are linked to the development of osteolytic bone lesions [9]. CCL3-positive cells are increased with increasing severity of periodontal disease and MCP-1/CCL2, CCL3, and CCL4 are present in periapical granulomas [10]. The capacity of CCL3 to promote bone resorption has been shown to occur through RANKL dependent and RANKL independent pathways and has recently been linked to suppression of coupled bone formation in leukemia [11]. Interestingly, RANKL also induces the production chemokines, suggesting an amplification loop during recruitment of precursors and differentiation of osteoclasts [12]. Elevated levels of SDF1/CXCL12 in the synovial and bone tissue of patients with rheumatoid arthritis are correlated to pathological bone loss caused by an increase in the recruitment and activation of osteoclasts at sites of local inflammation [13]. Collectively, these studies indicate a relationship between chemokine expression and osteoclastic bone resorption.

We have reported previously that impaired diabetic fracture healing is associated with elevated TNF- levels and osteoclast numbers [14]. Moreover, inhibition of TNF decreases diabetes-enhanced cartilage degradation and osteoclastogenesis [15, 16]. In the current study we examined chemokine expression in diabetic fracture repair and the role of the FOXO1 transcription in mediating TNF induced chemokine and chemokine receptor mRNA levels *in vitro*. The results show that diabetic fracture healing is associated with elevated levels of chemokines. CCL3 was examined by immunohistochemistry and shown to be expressed at higher levels in diabetic fractures, predominantly in hypertrophic chondrocytes in a TNF dependent manner. *In vitro* experiments using BMP stimulated ATDC5 and C3H10T1/2 cells with a hypertrophic chondrocyte phenotype demonstrated that FOXO1 knockdown decreased the expression of chemokines that were upregulated by TNF stimulation. Due to the capacity of chemokines to enhance inflammation through stimulation and activation of leukocytes and osteoclastogenesis, the results point to the possible involvement of chemokines in impaired diabetic fracture repair.

Material and Methods

Induction of Type 1 Diabetes and Femoral fracture

All experiments were conducted in conformity with Federal and USDA guidelines and had Institutional Animal Care & Use Committee (IACUC). Eight week old male CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA). Diabetes was induced by intraperitoneal injection of streptozotocin (40mg/kg) (Sigma, St. Louis, MO) daily for 5 days [17]. A group of mice were treated with vehicle alone (10 mM citrate). Evaluation of blood glucose levels was performed using blood samples taken from the tail (Accu-Chek, Roche Diagnostics, Indianapolis, IN). When the blood glucose levels exceeded 250 mg/dl mice were considered diabetic. Transverse closed fractures of the femur were performed in diabetic mice that were hyperglycemic for 3 weeks as described in [14, 17-19]. Fixation was achieved by placement of a 27 gauge spinal needle into the marrow cavity of the femur and fracture was induced by blunt trauma. Intraperitoneal injection of TNF inhibitor

pegsunercept (4mg/kg) was undertaken starting on day 10 post fracture and repeated every 3 days until euthanasia. Animals were euthanized at the 10 day and 16 day time points after fracture. Glycosylated hemoglobin level was measured by Glyco-tek affinity chromatography (Helena Laboratories, Beaumont, TX) at the time of euthanasia. Results showed no significant differences between pegsunercept treated and vehicle treated groups (data not shown).

Histology and Immunohistochemistry

Fixation of the specimens in cold 4% paraformaldehyde was performed for 72 hours followed by decalcification in cold Immunocal (Decal Corporation, Congers, NY) for 2 weeks. Embedding in paraffin and sectioning were performed as described in [18]. Deparaffinization and antigen retrieval was performed in 10 mM sodium citrate (pH 6.0) at 95 degree for 5 minutes. Specimens then were incubated with 3% hydrogen peroxide for 15 minutes at room temperature. Blocking was done using avidin biotin blocking system (Vector Laboratories, Burlingame, CA) and non-immune serum matching the secondary antibody. Incubation with anti MIP-1 /CCL4 antibody purchased from (R&D System) or matched non-specific IgG at 4C degree overnight was carried out, followed by several washing and incubation with biotin labeled secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Detection and visualization were done using an avidin-biotin kit from Vector Laboratories, and chromogen 3, 3'-diaminobenzidine (Zymed Laboratories Inc, South San Francisco, CA) and counterstained with hematoxylin. The percentage of expression of MIP-1 /CCL4 in different cell types in the callus was done taking 20 representative fields per callus using the scale shown in Supplemental Table 1. There were 6-7 samples per group. Analysis was done blindly by one examiner with the results confirmed by second examiner.

mRNA Profiling of Fracture Calluses

Fracture calluses from each group were collected, soft tissue was gently removed and specimens were snap frozen in liquid nitrogen. RNA extraction from each callus was performed using Trizol (Life Technologies, Rockville, MD) and the extracted RNA was purified using RNeasy MinElute cleanup kit (Qiagen, Valencia, CA). mRNA profiling was carried out using a PGA Mouse v1.1 array as we have previously described [15, 20]. Preparation of microarray probe and reading of fluorescent intensity were performed by the Massachusetts General Hospital Microarray Core Facility (Cambridge, MA). Data represent the mean of four replicates. The data related to chemokines and their receptors were analyzed and the difference in expression between diabetic and normoglycemic was analyzed as fold change.

FOXO1 RNAi and Overexpression

ATDC5 were cultured as we have previously described [15]. Cells were then plated in 6 well plates and when reached 70% confluency were transfected with 5nM ON-TARGETplus SMARTpool siRNAs against FOXO1 or control siRNA (Dharmacon, Chicago, IL) with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) in media supplemented with FBS (0.25%) as we have previously described [15]. Supernatant was collected and total RNA was extracted. Real time PCR was performed using probes and primer sets designed by the Universal Probe Library Assay Design Center (Roche, Indianapolis). Results were normalized by reference to mRNA levels of the housekeeping gene L32. mRNA profiling was carried out with a focused Chemokines and Receptors PCR Microarray (SA Biosciences, Valencia, CA) according to the manufacturer's instructions using the PCR Array Data Analysis web portal (SA Biosciences). Data represent the mean values obtained from three separate replicates. For overexpression of FOXO1 constructs containing the full length human FOXO1, constitutively active FOXO1AAA that was mutated at the Akt

phosphorylation sites or vector containing green fluorescent protein (GFP) were purchased from Addgene (Cambridge, MA). Transfections were performed using SuperFect (Qiagen). Cell lysates were examined for mRNA levels of CCL3 and CCL4 and supernatants were examined for protein level of CCL4 by ELISA (R&D Systems, Minneapolis, MN).

Statistical Analysis

Data are presented as mean values \pm SEM. Statistical significance between multiple groups for a given parameter was analyzed by ANOVA with Scheffe's post-hoc test. Results from immunohistochemistry using a scale from 0 to 5 were analyzed using Kruskal-Wallis non-parametric analysis of multiple groups and the Mann-Whitney post-hoc test. RNAi experiments and FOXO1 over-expression experiments were performed two to three times with similar results. In microarray data, statistical difference between diabetic and normoglycemic groups were evaluated using Student's T test ($p < 0.05$).

Results

To investigate the effect of diabetes on the expression of chemokines and chemokine receptors, microarray analysis was carried out using RNA samples from fracture calluses focusing on the stage of transition from cartilage to bone, day 16 following fracture. The difference in gene expression between diabetic and normoglycemic groups was considered significant if the P value was less than 0.05. Diabetic specimens showed upregulation of several chemokines and their receptors (Table 1) with a significant 1.28 to 5.71 fold increase in mRNA levels in more than 10 chemokines and receptors in diabetic compared to normoglycemic mice ($p < 0.05$). Several belonged to the CC subfamily of chemokines such as CCL4, CCL8, CCL6, CCL11, CCL20, CCL24 and the CC chemokine receptor CCR5 receptor. There was also a significant increase in the expression of CXC chemokines in the fracture sites of diabetic mice in vivo when compared to normoglycemic mice such as CXCL2, CXCL5 and the CXC chemokine receptor CXCR4.

Since CCL4 expression has been linked to osteoclastogenesis in inflammatory bone diseases [21] and was significantly up-regulated in the fracture calluses (Table 2) we further examined its expression in fracture calluses by immunohistochemistry. The percentage of CCL4 positive hypertrophic and proliferative chondrocytes was assessed in Figures 1A and 1B, respectively. In the normoglycemic group there was little increase in the expression of CCL4 in hypertrophic chondrocytes from day 10 to day 16, while there was in the diabetic group ($P < 0.05$; Figure 1). The increase in the diabetic group was blocked by inhibition of TNF ($P < 0.05$). The percent hypertrophic chondrocytes that expressed CCL4 was higher than proliferative chondrocytes in diabetic and normoglycemic groups on both days 10 and 16 ($P < 0.05$; Figure 1A compared to 1B). Examples of CCL4 positive chondrocytes are shown in Supplemental Figure 1. Semi-quantitative analysis was also performed to examine the relative expression of CCL4 in various cell types including proliferative chondrocytes, hypertrophic chondrocytes, osteoblasts/mesenchymal cells in developing bone and fibroblastic cells in the callus capsule using the scale shown in Supplemental Table 1. Overall hypertrophic chondrocytes had the highest level of CCL4 immunopositive cells followed by proliferative chondrocytes, osteoblastic/mesenchymal cells in the developing bone, and lastly, fibroblastic cells in the capsule (Figure 2). There was no difference between diabetic and normoglycemic mice in the percent positive CCL4 osteoblasts/mesenchymal cells or fibroblastic cells and the specific inhibition of TNF had no effect ($P > 0.05$).

In vitro studies were carried out to investigate a potential mechanism by which TNF may stimulate chemokines in ATDC5 chondrocytes. CCL4 was examined because of results obtained in Fig 1 and CCL3 was examined because of its role in promoting

osteoclastogenesis [22]. Cells were transfected with FOXO1 siRNA or scrambled siRNA and stimulated with TNF- α (20ng/ml). TNF induced a 4 fold increase in CCL3 and CCL4 mRNA levels. FOXO1 siRNA had little effect on CCL3 basal mRNA levels but did block almost all of the increase stimulated by TNF- α ($P<0.05$) (Fig 3). Under basal conditions FOXO1 knockdown reduced CCL4 mRNA and protein levels by approximately 50%. FOXO1 siRNA blocked almost all of the increase in CCL4 mRNA stimulated by TNF- α . TNF- α induced a 2 fold increase of CCL4 at the protein level, which was blocked by FOXO1 knockdown ($P<0.05$; Figure 3C). In contrast scrambled siRNA had no effect on CCL3 or CCL4 expression ($P>0.05$).

Additional experiments were carried out examining ATDC5 chondrocytes transfected with the full length human FOXO1 or FOXO1AAA that has been mutated so that it is constitutively active [23]. Transfection with constitutively active FOXO1AAA induced ~3 fold increase in CCL3 mRNA and FOXO1 and FOXO1AAA induced a 3 fold increase in CCL4 mRNA levels. When released CCL4 was measured at the protein level FOXO1 induced a 50% increase and FOXO1AAA a 100% increase. Transfection with vector containing green fluorescent protein had no effect.

To further explore the role of TNF- α in chemokine induction in ATDC5 chondrocytes, *in vitro* experiments were performed using a focused PCR array (Table 2). TNF- α significantly enhanced mRNA levels of 14 out of 25 chemokines in the array (56%) with the degree of upregulation ranging from 6 to greater than 1000 fold ($P<0.05$) (Table 2). In every case, FOXO1 knockdown significantly ($P<0.05$) blunted the increase stimulated TNF- α (Table 2). In addition 19 chemokine receptors were examined for increased expression by TNF- α and regulation by FOXO1. Two of these were significantly increased by TNF and two others just missed being significant. mRNA levels for CCR1, CCRL2 and CCR4 were significantly reduced by FOXO1. Other inflammatory genes and morphogenetic proteins genes included in the array were also upregulated by TNF stimulation by at least 6 fold (Table 2B; $P < 0.05$). In each case the TNF mediated increase was reduced by FOXO1 knockdown. These results point to the overall importance of TNF in induction of inflammatory molecules including chemotactic cytokines and their receptors in chondrocytes and the possible role of FOXO1 in mediating the effect of TNF stimulation.

Discussion

Diabetes has a significant impact on fracture healing and increases the inflammatory environment at the fracture site [14, 24, 25]. By mRNA profiling we show here a potentially unrecognized mechanism for diabetes impaired fracture healing; enhanced expression of chemokines during the transition from cartilage to bone in the healing fracture callus. Diabetic fracture sites had significant upregulation of several chemokines and receptors, CCL4, CCL8, CCL6, CCL11, CCL20, CCL24, CXCL2, CXCL5 and chemokine receptors CCR5 and CXCR4. *In vitro* TNF- α stimulated mRNA levels of several chemokines in chondrocytes that were also enhanced by diabetes *in vivo* including CCL4, CCL8, CCL20, CXCL2 and CXCL5. Each of these is chemotactic for leukocyte subsets including lymphocytes, monocytes and PMNs and to be produced by chondrocytes [26-28]. The induction of chemokines by TNF- α is consistent with previous findings that the hyperinflammatory response in diabetic fracture healing is caused in part, by the elevated levels of TNF- α [15,16]. Moreover, CCL4 was shown by immunohistochemistry to be induced in hypertrophic chondrocytes in diabetic fracture sites by a TNF-dependent mechanism. Two chemokines upregulated by diabetes *in vivo* CCL6 and CCL11 were not regulated by TNF *in vitro*, suggesting that they may be regulated by other factors present in diabetic calluses or primarily expressed in cells other than chondrocytes. Compared to chemokine ligands there was less overlap between chemokine receptors elevated in diabetic

fracture calluses and regulation of chemokine receptors by TNF *in vitro*. This may reflect the relatively few chemokine receptors that were included in the *in vivo* microarray or expression by cells other than chondrocytes. However, expression of CCR1 and CCR4 have been found in chondrocytes [29, 30] and were regulated by TNF in a FOXO1 dependent manner.

In summary, we report here that diabetic fracture calluses are characterized by enhanced chemokine expression and at least in some cases, chondrocytes are likely to be an important source as shown for CCL4. The expression of chemokines could contribute to the accelerated loss of cartilage noted in diabetic fracture calluses [14, 17] by enhancing the level of inflammation through recruitment of leukocyte subsets or through the recruitment of osteoclast precursors and osteoclast formation [26]. The potential importance of inflammatory mediators is underscored by the rapid loss of cartilage under conditions where inflammation is enhanced such as diabetes [14, 31]. Conversely, when inflammation is suppressed by genetic deletion of TNF receptors, degradation of cartilage is delayed [32]. This may be significant since cartilage forms the anlagen for endochondral bone formation. In addition, chemokines have other potential effects such as the production of degradative enzymes and autocrine stimulation of osteoclasts that may enhance apoptosis [26, 33]. Thus, it is possible that dysregulation in diabetes leads to elevated production of cytokines such as TNF- α , which in turn induces the expression of chemokines that amplify the level of inflammation and catabolic effect of diabetes on diabetic fracture healing.

Chemokines and their receptors have been found to participate in diseases involving bone and cartilage degradation such as osteoarthritis and rheumatoid arthritis and osteolytic lesions from various pathologies [4, 34, 35]. Thus, it is possible that chemokine production by chondrocytes has an autocrine or paracrine role in including an effect on inflammation, apoptosis, production of degradative enzymes and formation of osteoclasts [36]. Experiments with chemokine inhibition at the time of cartilage degradation would be needed to definitively establish the role of chemokines in the transition from cartilage to bone during fracture healing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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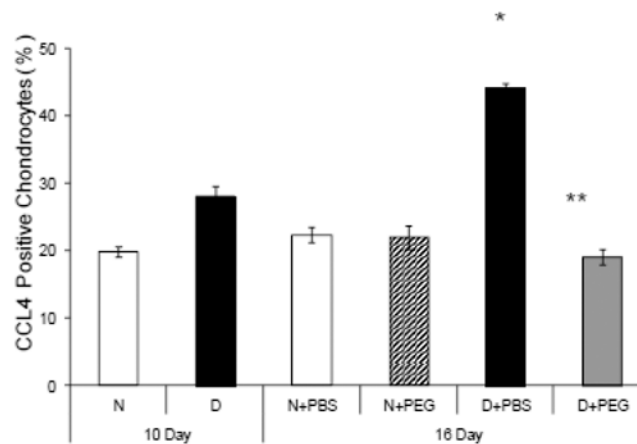
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Highlights

- Diabetes enhances chemokine expression in Fracture Healing
- Hypertrophic chondrocytes express chemokines during fracture healing in a TNF dependent manner
- TNF induced chemokine expression in chondrocytes is mediated by transcription factor FOXO1

A



B

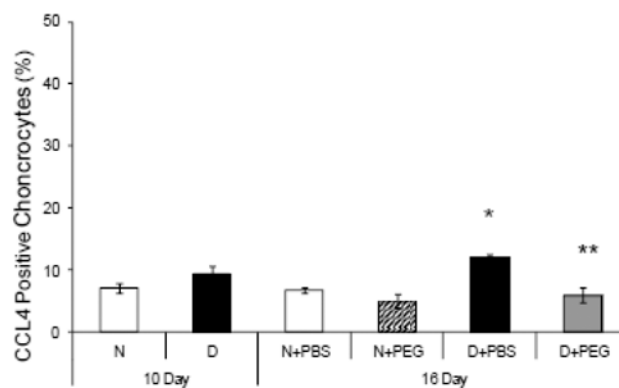


Figure 1. Chondrocytes express enhanced CCL4 expression in diabetic fractures that is TNF dependent

Femoral fractures were induced in normoglycemic (N) or diabetic (D) mice which were treated with the TNF-specific inhibitor, pegsunercept (PEG) or PBS alone (PBS) starting on day 10. Transverse sections were examined by immunohistochemistry for expression of CCL4. Figure 1A: The percent hypertrophic chondrocytes that expressed CCL4. Figure 1B: The percent proliferative chondrocytes that expressed CCL4. * indicate significant differences between normal and diabetic groups ($P < 0.05$). ** Indicates differences between diabetic and PEG treated groups ($P < 0.05$).

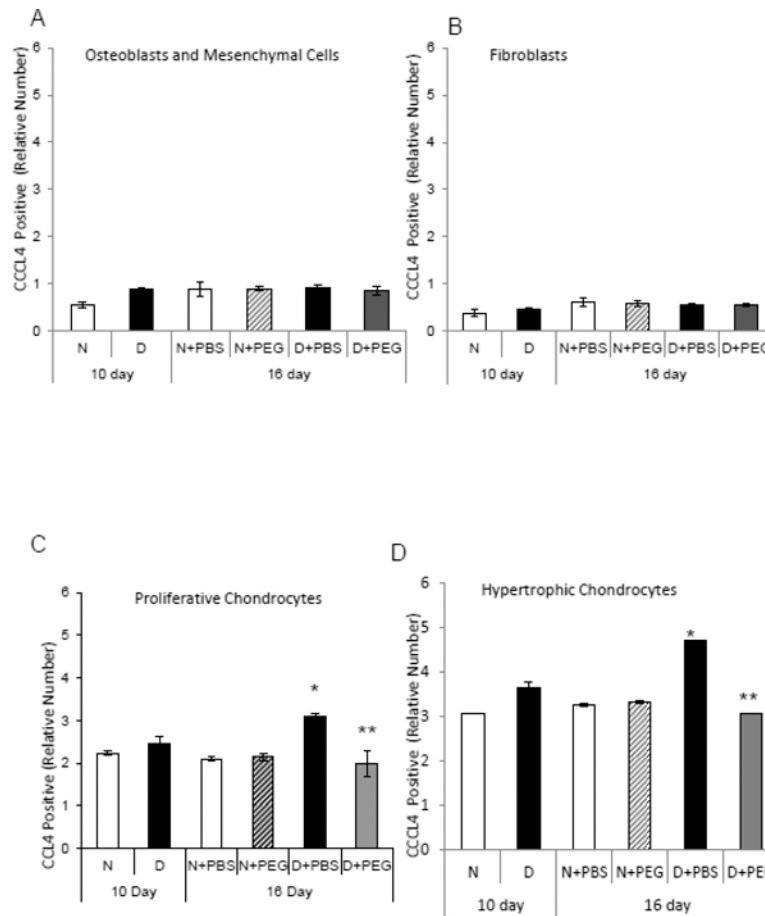


Figure 2. CCL4 expression occurs preferentially in hypertrophic chondrocytes

Sections from fracture calluses were examined by immunohistochemistry for CCL4 expression. The relative percent CCL4 positive cells was assessed from a scale of 0 to 5 as described in Supplemental Table 1. A, Osteoblasts and mesenchymal cells; B, Fibroblasts; C, Proliferative chondrocytes; and D, Hypertrophic chondrocytes. Significance was determined by Kruskal–Wallis one-way analysis of variance. * Indicates significant difference between normal and diabetic groups ($p < 0.05$). ** Indicates significant differences between 16-day diabetic and diabetic pegsunercept treated groups ($p < 0.05$).

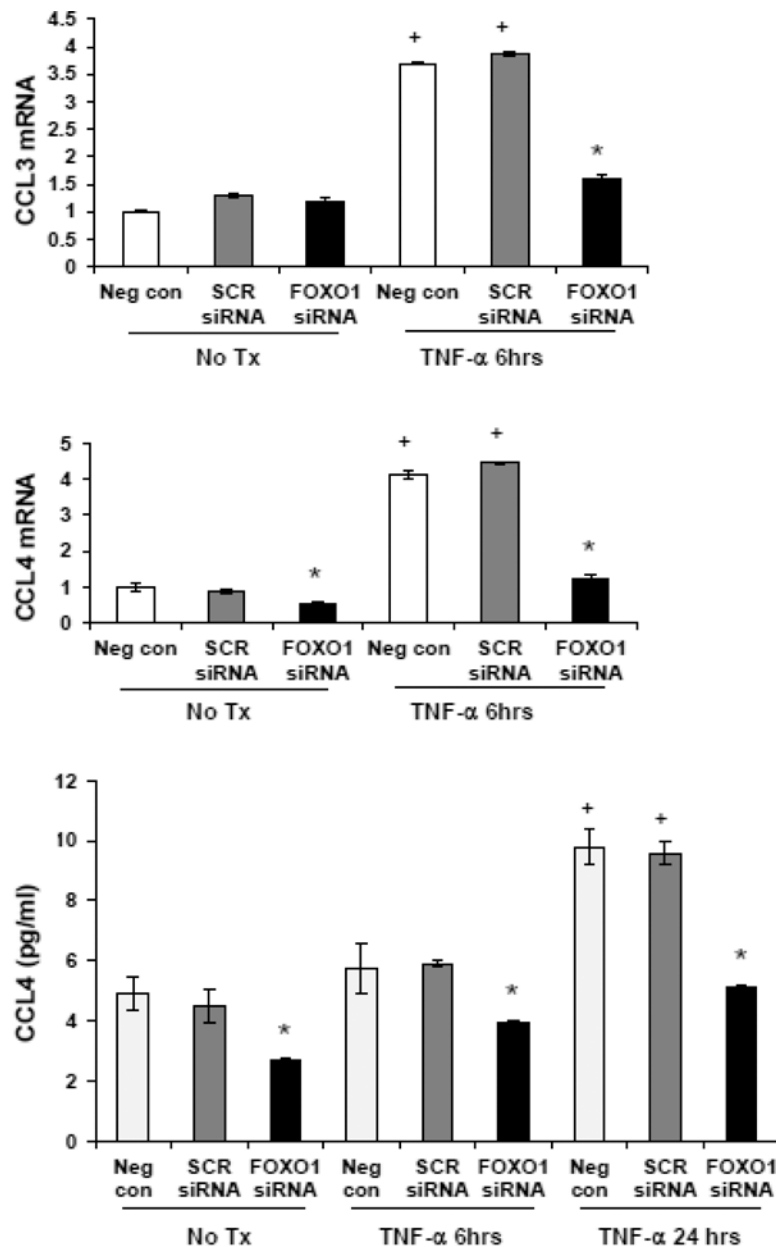


Figure 3. TNF induced CCL4 is FOXO1 dependent in chondrocytes

ATDC5 chondrocytes were transfected with FOXO1 siRNA or scrambled siRNA and stimulated with TNF- α (20ng/ml). mRNA levels of CCL3 or CCL4 were measured by real-time PCR and the protein level of CCL4 was measured by ELISA. mRNA levels are expressed relative to baseline. Data are representative of three independent experiments. + indicates significantly higher than baseline control ($P < 0.05$); * indicates a significantly less than matched scrambled siRNA ($P < 0.05$).

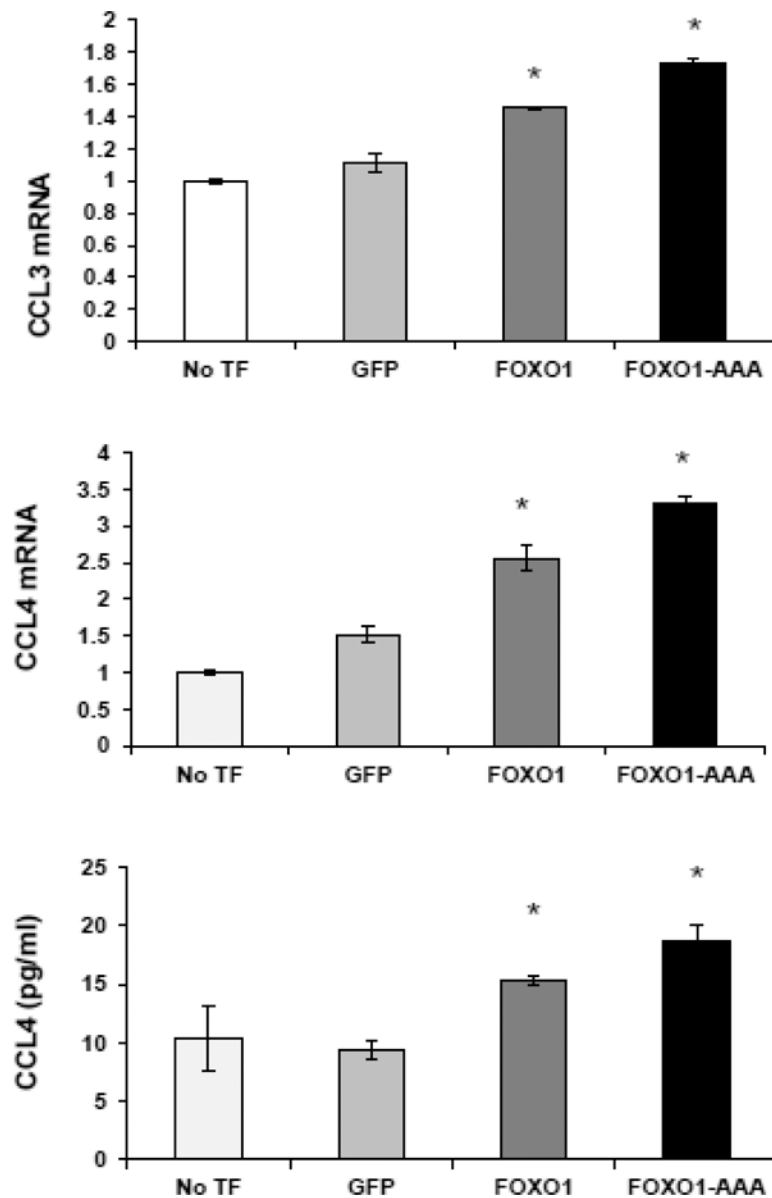


Figure 4. FOXO1 over-expression induces CCL3 and CCL4 in chondrocytes

ATDC5 cells were transfected with constructs containing GFP (negative control), FOXO1 or constitutively active FOXO1AAA. Cell lysates were examined for mRNA levels of CCL3 or CCL4 by real-time PCR and the protein level of CCL4 was measured by ELISA. Data are representative of two or three independent experiments. * indicates significantly higher than GFP negative control ($P < 0.05$).

Table 1
Chemokines and chemokines Expression from Microarray analysis in fracture calluses

Chemokine	Fold Change (Diab/Norm)	P value	Chem Recep	Fold Change (Diab/Norm)	P value
Ccl1	3.06	0.84	Ccr1	1.22	0.14
Ccl2	0.96	0.67	Ccr1	1.22	0.14
Ccl3	0.94	0.56	Ccr2	2.56	0.63
Ccl4	2.66	0.02*	Ccr3	1.02	0.96
Ccl5	1.38	0.15	Ccr5	1.80	0.02*
Ccl6	2.36	0.03*	Ccr6	1.91	0.30
Ccl8	3.64	0.01*	Ccr7	0.66	0.13
Ccl9	1.14	0.70	Cxcr4	1.44	0.03*
Ccl11	2.19	0.02*	Cx3cr1	1.20	0.50
Ccl12	2.77	0.08			
Ccl17	1.46	0.30			
Ccl19	1.13	0.80			
Ccl20	5.71	0.020*			
Ccl21b	1.28	0.03*			
Ccl22	1.06	0.72			
Ccl24	1.79	0.03*			
Ccl25	0.93	0.57			
Ccl28	0.75	0.24			
Cxel1	1.23	0.28			
Cxel2	4.89	0.00*			
Cxel4	1.66	0.19			
Cxel5	3.12	0.00*			
Cxel7	1.03	0.91			
Cxel9	0.78	0.14			
Cxel10	1.02	0.80			
Cxel11	1.84	0.39			

Chemokine	Fold Change (Diab/Norm)	P value	Chem Recep	Fold Change (Diab/Norm)	P value
Cxcl12	0.96	0.76			
Cxcl13	1.68	0.93			
Cxcl14	1.23	0.61			
Cxcl15	1.19	0.56			
Cxcl16	1.35	0.54			
Cx3cl1	1.63	0.06			
Xcl1	1.17	0.58			

* indicates significant differences between diabetic and normoglycemic group.

Table 2 A
Chemokines and Receptors Gene Expression from PCR Focused Microarray in ATDC5 cells with hypertrophic chondrocytes

Chemokine	TNF- Treatment		FOXO1 knockdown	
	Fold Change	P value	Fold Change	P value
Ccl1	6.13	0.04	0.19	0.05
Ccl2	>1000	0.02	0.05	0
Ccl4	29.6	0.05	0.03	0
Ccl5	>1000	0.04	0.02	0
Ccl6	0.94	0.99	0.06	0.02
Ccl7	95.4	0	0.04	0
Ccl8	12.44	0	0.29	0
Ccl9	212.2	0	0.04	0
Ccl11	5.86	0.3	0.24	0.2
Ccl12	0.15	0.49	2.5	0.4
Ccl17	59.28	0	0.07	0
Ccl19	0.37	0.78	0.25	0.43
Ccl20	>1000	0	0.03	0
Cxcl1	12.55	0.02	0.3	0
Cxcl2	48.74	0	0.11	0
Cxcl5	11.34	0	0.17	0
Cxcl9	>1000	0	0	0
Cxcl10	>1000	0	<0.001	0
Cxcl11	82.1	0.32	<0.001	0
Cxcl12	2.78	0.57	2.36	0
Cxcl13	88.1	0.1	1.14	0.86
Cxcl15	3.31	0.56	0.13	0.06
Cx3cl1	724.28	0.08	0.07	0
Xcl1	124.3	0.58	0.02	0.61
Chemokine Receptor	Fold Change	P value	Fold Change	P value
Ccr1	4.46	0.05	0.23	0.05
Ccr2	0.83	0.9	0.99	0.99
Ccr3	2.31	0.54	0.2	0.03
Ccr4	7.43	0.07	0.74	0.04
Ccr5	0.3	0.68	0.4	0.28
Ccr6	40.15	0.09	1.06	0.89
Ccr7	1.69	0.44	0.75	0.39
Ccr8	0.44	0.71	0.47	0.23
Ccr9	1.81	0.43	1.2	0.61
Ccr10	3.86	0.56	11.14	0.19
Ccr11	0.32	0.4	2.28	0.14
Ccr12	653.19	0	0.01	0
Ccr111	1.35	0.8	0.85	0.89

Chemokine	TNF- Treatment		FOXO1 knockdown	
	Fold Change	P value	Fold Change	P value
Cxcr3	0.95	0.99	0.7	0.61
Cxcr4	0.52	0	3.33	0
Cxcr5	4.28	0.06	0.81	0.73
Cxcr6	1.35	0.28	1.22	0.07
Cxcr7	0.67	0.87	0.5	0
Cx3cr1	0.2	0.71	0.93	0.96

Table 2 B
Other Inflammatory Gene Expression from PCR Focused Microarray in ATDC5 cells
with hypertrophic chondrocytes

	TNF- Treatment		FOXO1 knockdown	
	Fold Change	P value	Fold Change	P value
TNFa	>1000	0.00	<0.001	0.00
Inhbb	>1000	0.25	<0.001	0.32
Csf1	102.28	0.15	0.16	0.00
Nfkb1	49.74	0.19	0.31	0.00
Cmtm4	45.88	0.24	0.78	0.03
Bmp10	37.51	0.02	0.03	0.01
Hif1a	24.80	0.35	0.95	0.70
Il1a	23.53	0.00	0.18	0.00
Lif	17.85	0.42	0.67	0.89
Tnfrsf1a	14.70	0.31	0.98	0.79
Tlr4	14.10	0.26	1.07	0.82
Ppbp	13.78	0.30	0.70	0.00
Il18	11.77	0.13	0.77	0.00
Tnfsf14	7.88	0.18	0.16	0.23
Cmtm6	6.02	0.03	0.59	0.00
Bdnf	5.66	0.18	0.64	0.04
Csf2	5.09	0.62	0.29	0.00
Mmp2	3.46	0.12	1.10	0.47
Il13	3.39	0.37	1.49	0.62
Rgs3	2.81	0.10	0.56	0.46
Il4	2.77	0.32	1.46	0.44
Bmp6	1.83	0.36	0.75	0.12
Myd88	1.51	0.78	0.32	0.00
Il8ra	1.47	0.29	2.09	0.19
Inha	1.27	0.77	3.36	0.00
Tymp	1.21	0.16	1.94	0.01
Cmtm3	1.09	0.90	0.96	0.66
Cmklr1	0.88	0.91	1.59	0.04
Il8rb	0.50	0.83	0.15	0.09
Trem1	0.39	0.41	1.48	0.63
Agtrl1	0.22	0.68	0.35	0.43
Bmp15	0.18	0.31	4.48	0.08
Ccbp2	0.18	0.09	3.74	0.17
Pf4	0.16	0.63	0.52	0.13
Gdf5	0.11	0.23	8.19	0.08
Cmtm5	0.05	0.45	1.48	0.63
Gpr81	0.05	0.53	0.54	0.47
Il16	0.05	0.41	0.56	0.64

	TNF- Treatment		FOXO1 knockdown	
	Fold Change	P value	Fold Change	P value
Slit2	0.05	0.41	0.82	0.47
Ltb4r2	0.03	0.32	2.38	0.03
Cmtm2a	0.02	0.24	0.64	0.64

ATDC5 cells were transfected with FOXO1siRNA or scrambled siRNA and stimulated with TNF- as for 6 hour. The TNF- treatment columns give the mRNA values for cells incubated with TNF- plus scrambled siRNA divided by the value for scrambled siRNA and the p values calculated for these two groups. FOXO1 knockdown columns give the mRNA values for cells incubated with TNF- plus FOXO1 siRNA divided by the value for TNF- plus scrambled siRNA and the p values calculated for these two groups. Bolded results represent changes where there was at least 1.7 fold increase or 0.7 fold decrease and were statistically significant (P < 0.05).