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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 ($P<0.05$). 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.

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 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-α levels and osteoclast numbers [14]. Moreover, inhibition of TNF decreases diabetes-enhanced cartilage degradation and osteoclastogenesis [15,16]. In the current

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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. CCL4 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 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 (40 mg/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 (4 mg/kg) was undertaken starting on day10 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 h 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° for 5 min. Specimens then were incubated with 3% hydrogen peroxide for 15 min 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-1β/CCL4 antibody purchased from (R&D System) or matched non-specific IgG at 4 °C 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-1β/CCL4in 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 RNAeasy 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 5 nM 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

Table 1
Chemokines and chemokine 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**[a]	Ccr3	1.02	0.96
Ccl5	1.38	0.15	**Ccr5**	**1.80**	**0.02**[a]
Ccl6	**2.36**	**0.03**[a]	Ccr6	1.91	0.30
Ccl8	**3.64**	**0.01**[a]	Ccr7	0.66	0.13
Ccl9	1.14	0.70	**Cxcr4**	**1.44**	**0.03**[a]
Ccl11	**2.19**	**0.02**[a]	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**[a]			
Ccl21b	**1.28**	**0.03**[a]			
Ccl22	1.06	0.72			
Ccl24	**1.79**	**0.03**[a]			
Ccl25	0.93	0.57			
Ccl28	0.75	0.24			
Cxcl1	1.23	0.28			
Cxcl2	**4.89**	**0.00**[a]			
Cxcl4	1.66	0.19			
Cxcl5	**3.12**	**0.00**[a]			
Cxcl7	1.03	0.91			
Cxcl9	0.78	0.14			
Cxcl10	1.02	0.80			
Cxcl11	1.84	0.39			
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			

Bold indicates a significant difference in mRNA level between normal and diabetic on day 16 ($P<0.05$).
[a] Indicates significant differences between diabetic and normoglycemic group.