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TNFα contributes to diabetes impaired angiogenesis in fracture healing

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ABSTRACT

Diabetes increases the likelihood of fracture, interferes with fracture healing and impairs angiogenesis. The latter may be significant due to the critical nature of angiogenesis in fracture healing. Although it is known that diabetes interferes with angiogenesis the mechanisms remain poorly defined. We examined fracture healing in normoglycemic and streptozotocin-induced diabetic mice and quantified the degree of angiogenesis with antibodies to three different vascular markers, CD34, CD31 and Factor VIII. The role of diabetes-enhanced inflammation was investigated by treatment of the TNFα-specific inhibitor, pegsunercept starting 10 days after induction of fractures. Diabetes decreased both angiogenesis and VEGFA expression by chondrocytes. The reduced angiogenesis and VEGFA expression in diabetic fractures was rescued by specific inhibition of TNF *in vivo*. In addition, the TNF inhibitor rescued the negative effect of diabetes on endothelial cell proliferation and endothelial cell apoptosis. The effect of TNFα *in vitro* was enhanced by high glucose and an advanced glycation endproduct to impair microvascular endothelial cell proliferation and tube formation and to stimulate apoptosis. The effect of TNF, high glucose and an AGE was mediated by the transcription factor FOXO1, which increased expression of p21 and caspase-3. These studies indicate that inflammation plays a major role in diabetes-impaired angiogenesis in endochondral bone formation through its effect on microvascular endothelial cells and FOXO1.

1. Introduction

Diabetes impairs bone formation and delays fracture healing, presenting a challenge in the management of diabetic fractures. Diabetics also have more complications during fracture healing [1–3]. Fracture healing requires angiogenesis and an adequate blood supply [4,5]. Interference with angiogenesis impairs fracture healing [6,7] while treatment with angiogenic factors such as VEGFA and FGF-2 improves bone formation during repair [8,9]. Diabetes leads to reduced angiogenesis that may contribute to the pathologic outcomes in diabetic complications [10]. Endothelial progenitor cells are significantly reduced in types 1 and 2 diabetic patients and in animal models [11]. Injection of $CD34^+$ endothelial progenitors in diabetic mice augments vascularization and improves wound healing [12] and the application of angiogenic factors promotes vascularization and diabetic fracture healing in a rat model [13]. Although it is known that diabetes reduces angiogenesis in fracture repair the mechanisms for this decrease have not been established [13].

Diabetes has been shown to increase inflammation in fracture calluses that promotes early cartilage resorption and reduced mesenchymal stem cell numbers [14,15]. The early inflammatory response is beneficial by inducing recruitment of mesenchymal stem cells and leukocytes, which produce factors to stimulate tissue repair and angiogenesis [16]. However, prolonged inflammation leads to deficient bone formation and impaired fracture healing [17–19]. In particular, tumor necrosis factor-alpha (TNFα) levels remain elevated in diabetic fractures, resulting in early apoptosis of chondrocytes and mesenchymal stem cells [15,20]. Thus, elevated levels of TNFα later in fracture repair may contribute to poor fracture healing in diabetics.

In the current study, we demonstrate that diabetes hampers angiogenesis in areas of endochondral ossification during fracture healing and reduces VEGFA expression. Specific inhibition of TNFα with pegsunercept after the early phase restored blood vessel formation in diabetic fractures. Mechanistically, the TNFα-dependent changes were

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due to reduced expression of VEGFA and reduced proliferation and increased apoptosis of endothelial cells. The latter were mediated *in vitro* by the transcription factor FOXO1. Our findings indicate that the vascular deficit associated with fracture healing is due in part to diabetes-enhanced TNFα and that the control of inflammation during fracture repair may offer a pragmatic approach to augment diabetic fracture healing.

2. Materials and methods

2.1. Animals

Diabetes was induced in 8-week-old male CD-1 male mice (Charles River Laboratories, Wilmington, MA) with a daily intraperitoneal injection of streptozotocin (STZ, 40 mg/kg, Sigma-Aldrich, St. Louis, MO) for 5 days. Control mice were treated with vehicle alone (10 mM citrate buffer). Mice were considered hyperglycemic when blood glucose levels were greater than 12.5 mmol/l. STZ-induced diabetic mice received insulin treatment through insertion of slow release insulin implants as described previously [21] or i.p injection of pegsunercept, a TNF specific inhibitor as described below. A simple transverse closed fracture of the tibia (insulin studies) or femur (TNF inhibitor studies) was performed as previously described [21]. The articular surface of the tibia or femur was exposed and a 27-gauge spinal needle was inserted for fixation. After closure of the incision a fracture was created by blunt trauma. Any fractures not consistent with standardized placement criteria (mid-diaphyseal) or grossly comminuted were excluded. Animals were subsequently euthanized at the indicated time points. Bone was harvested with most of the muscle and soft connective tissue was removed. Mice were hyperglycemic for at least 3 weeks prior to fracture. In some experiments, animals were treated with TNF-α inhibitor pegsunercept (4 mg/kg, Amgen, Thousand Oaks, CA) by intraperitoneal

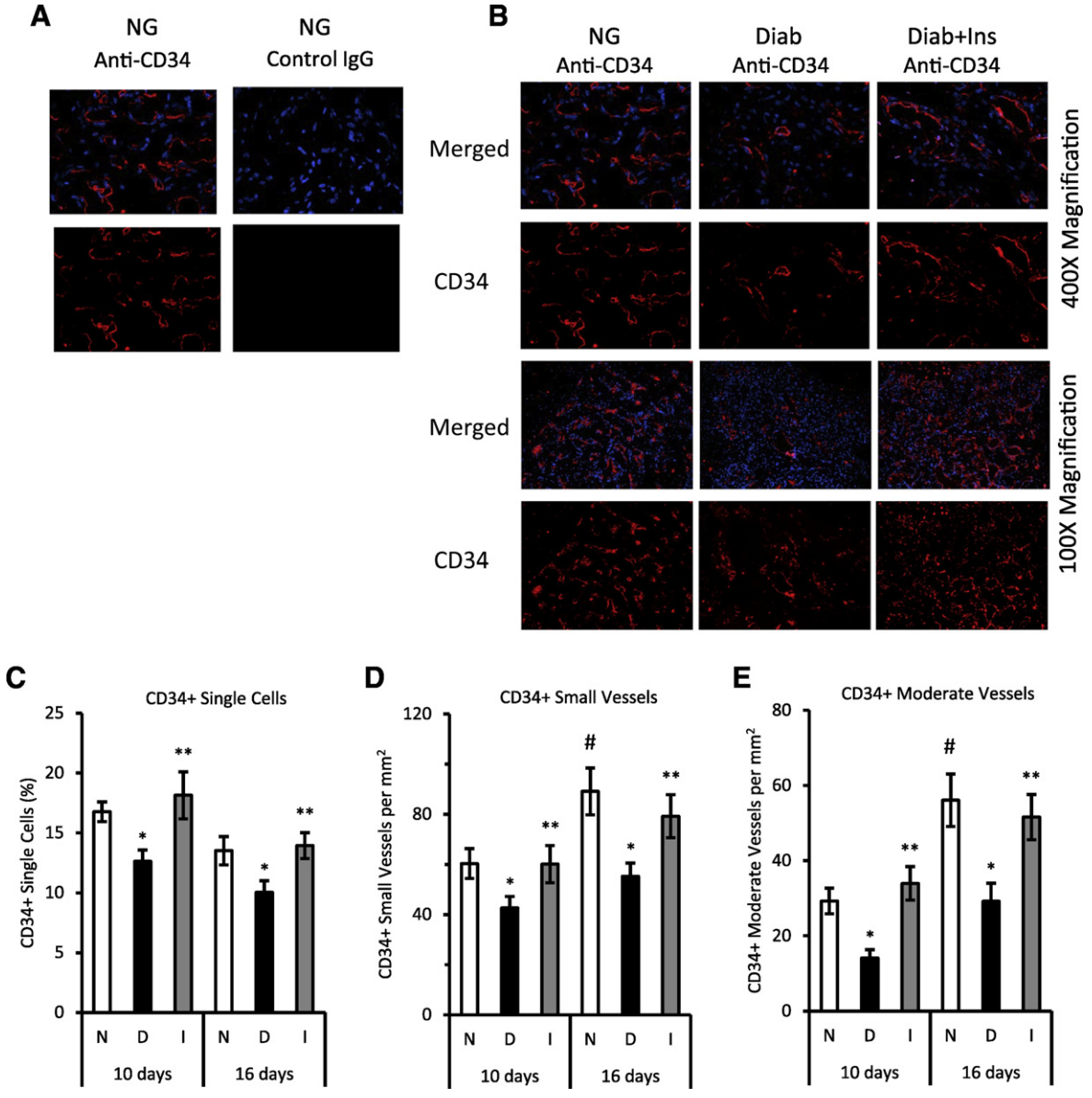

Fig. 1. Diabetes decreases CD34$^+$ and Factor VIII$^+$ blood vessels in areas of endochondral bone formation during fracture repair. A: Immunofluorescent images following incubation with anti-CD34$^+$ IgG or matched control IgG in normoglycemic mice and B: immunofluorescent images of CD34$^+$ expression in normoglycemic (N), diabetic (D) and insulin-treated diabetic (I) mice from areas of bone formation in 16 day fracture specimens (400× or 100× magnification). C–E: CD34 immunopositive single cells, small vessels or moderate-sized vessels were analyzed as described in the Methods at 10 and 16 days after fracture. F–G: Factor VIII positive blood vessels were detected by immunohistochemistry in areas of bone formation at 10 and 16 days post fracture. H–I: Factor VIII positive blood vessels were quantified in areas of mature bone at 22 days after fracture. N = 5–6 per group. *$p < 0.05$ between normal vs diabetic groups, **$p < 0.05$ between diabetic and insulin-treated groups, # $p < 0.05$ between corresponding groups on days 10 and 16.