Insulin-dependent diabetes impairs the inflammatory response and delays angiogenesis following Achilles tendon injury

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Submitted 17 September 2003; accepted in final form 19 December 2003

Chbinou, Nadia, and Jérôme Frenette. Insulin-dependent diabetes impairs the inflammatory response and delays angiogenesis following Achilles tendon injury. Am J Physiol Regul Integr Comp Physiol 286: R952–R957, 2004. First published January 8, 2004; 10.1152/ajpregu.00536.2003.—Although impaired wound healing associated with type 1 diabetes mellitus has been well studied in skin tissue, the influence of this metabolic disorder on tendon healing and recovery has not been extensively investigated. Because tendons are known to have limited repair potential, we studied the tendon-healing process by using a diabetic rat tendonitis model. We tested the hypothesis that diabetes influences the inflammatory response, cell proliferation, and angiogenesis in injured Achilles tendons. Diabetes was induced by injecting streptozotocin at 45 mg/kg body wt. Nondiabetic rats as well as diabetic and insulin-treated diabetic animals were then injected with collagenase. The accumulation of inflammatory cells was quantified in transversal sections of Achilles tendon by using immunohistochemical staining at days 0, 1, 3, 7, 14, and 28 posttrauma. The number of proliferative cells and the extent of neovascularization was also quantified in the paratenon and the core of the tendon at days 0, 3, 7, 14, and 28 posttrauma. Relative to nondiabetic and insulin-treated diabetic animals, the numbers of accumulated neutrophils and ED1+ and ED2+ macrophages in diabetic rats decreased by 46, 43, and 52%, respectively, in the first 3 days after injury compared with levels in nondiabetic and insulin-treated diabetic animals. The density of newly formed blood vessels decreased by 35 and 29% in the paratenon and the core of tendon, respectively, at days 3 and 7 after injury. Lastly, the concentration of proliferative cells decreased by 34% in the paratenon at day 7 posttrauma in injured tendons from diabetic rats relative to nondiabetic rats. These results indicate that alterations in inflammatory, angiogenic, and proliferative processes occurred in the diabetic state that might eventually perturb tendon healing and remodeling.

TYPE 1 DIABETES MELLITUS RESULTS FROM A DESTRUCTION OF PANCREATIC β-CELLS, WHICH INDUCES PARTIAL OR ABSOLUTE INSULIN DEFICIENCY. THIS METABOLIC DISEASE IS ASSOCIATED WITH A HIGH LEVEL OF GLYCEREMIA, WHICH LEADS TO MANY PATHOLOGICAL CHANGES, INCLUDING PERIPHERAL NEUROPATHY, KIDNEY AND GASTROINTESTINAL DYSFUNCTIONS, IMMUNODEFICIENCY, MICROVASCULAR LESIONS, AND TISSUE-REPAIR DISORDERS (24). CLINICAL AND EXPERIMENTAL STUDIES REVEALED THAT THE DIABETIC STATE IS OFTEN ASSOCIATED WITH ALTERATIONS IN ANGIOGENESIS, CELLULAR PROLIFERATION, THE INFLAMMATORY RESPONSE, AND, ULTIMATELY, IMPAIRMENT OF WOUND HEALING (7, 8, 11, 35). FOR EXAMPLE, SUSTAINED INDUCTION OF CHEMOKINES AND PROLONGED PERSISTENCE OF NEUTROPHILS (PMNs) AND MACROPHAGES (MΦs) WERE OBSERVED IN GENETICALLY DIABETIC db/db mice during the late phase of cutaneous repair (32). FURTHERMORE, THE NUMBER OF APOTOTIC CELLS INCREASED, WITH A CONCOMITANT REDUCTION OF FIBROBLAST MIGRATION IN DIABETIC MICE COMPARED WITH THEIR WILD-TYPE COUNTERPARTS, INDICATING THAT DIABETES AFFECTS A BROAD SPECTRUM OF BIOLOGICAL ACTIVITIES THAT ARE ESSENTIAL FOR WOUND HEALING (2, 13).

In tendons, the sequence and time course of events that occur during healing closely resemble those reported in many other tissues. The injured tendon is first filled by exudated fibronectin and fibrin, which form a scaffold for the migration of various cells and stimulate vascular ingrowth. Different subpopulations of leukocytes rapidly invade the inflamed tissue to phagocytose cellular debris and stimulate fibroblast proliferation and collagen deposition (6). This involves many different inflammatory cell populations that appear at distinct stages of the inflammatory process. We have shown that PMN populations are significantly elevated at days 1 and 3 after collagenase injury (17). The concentration of PMNs then declines, and apoptotic PMNs are probably phagocytosed by fibroblasts and MΦs (22). MΦ populations thereafter become the predominant cell subset until the end of the inflammatory process. The heterogeneity of leukocyte populations implies that different subclasses of leukocytes have complementary functions during tissue healing. For instance, ED1+ MΦs are likely involved in removing necrotic tissue by phagocytosis, whereas ED2+ MΦs are resident cells that can enhance cell proliferation (19).

Although few studies have been conducted on tendon healing and functional recovery in the diabetic state, human and animal models of diabetes have shown that intracellular degradation of procollagen (14) and morphological abnormalities occur in Achilles tendons (9). Other evidence also suggests that diabetic patients are more susceptible than healthy people to developing chronic tendinitis. Our overall goal was to elucidate the impact of diabetes on the inflammatory response, cell proliferation, and angiogenesis in tendon healing. We observed that the accumulation of PMNs and ED1+/ED2+ MΦs and the rate of proliferation of new vessels and tendon cells decreased in injured Achilles tendons from diabetic streptozotocin (STZ)-treated animals. In addition, the attenuation of hyperglycemia with insulin restored the inflammatory response and healing process in Achilles tendon. These results support the concept that diabetes may perturb wound healing by modulating the early phase of tendon repair.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 180–200 g (Charles River, QC, Canada) were housed two per cage, maintained on a 12:12-h dark-light schedule, and randomly assigned to four separate...
groups: 1) ambulatory control rats, 2) nondiabetic rats, 3) diabetic rats, and 4) insulin-treated diabetic rats. Control rats were not injected with collagenase or PBS. This study was approved by Université Laval’s Animal Research Committee.

**Induction of diabetes.** Animals were anesthetized with isoflurane, and diabetes was induced by an intravenous injection of STZ (Sigma, St. Louis, MO), a pancreatic cell toxin, at 45 mg/kg body wt. The STZ was dissolved in citrate buffer (pH 4.6) containing 73 mM citric acid, 150 mM NaOH, and 25 mM HCl. Four days after the STZ injection, STZ-treated and control (untreated) animals were fasted for 12 h and then submitted to a glucose tolerance test, which consists of measuring blood glucose levels 0, 15, 30, 60, 90, and 120 min after an intraperitoneal injection of a 50% dextrose solution in sterile saline at 1.5 g/kg body wt (Abbott Laboratories, Abbott Park, IL). STZ-injected rats were considered diabetic when 1) peak glucose values observed early in the glucose tolerance test were significantly higher than the mean value for nondiabetic rats or 2) glucose levels did not return to baseline values within the first 2 h. All diabetic rats had fasting blood glucose concentrations equal or superior to 12 mmol/l. Diabetic animals were then assigned to be treated or not with insulin to test the effect of insulin treatment on tendon repair. Treatment with insulin was adjusted such that diabetic rats had normal glycemia. Regular and ultralent insulin were injected subcutaneously in the animals at 0.5 units regular and 3 ± 0.5 units ultralent) and at night (3 ± 0.5 units regular and 5 ± 0.5 units ultralent) during the entire experimental protocol.

**Induction of tendon injury.** Six days after the injection of the STZ, control, diabetic, and insulin-treated diabetic animals were anesthetized with an intraperitoneal injection of ketamine-xylazine cocktail at 87.5 and 12.5 mg/kg body wt, respectively. The skin covering the Achilles tendons was shaved and disinfected. The rats were then injected subcutaneously with 5 ml of Ringer lactate to prevent dehydration. Right Achilles tendons were then percutaneously injected with 15 μl of a collagenase solution prepared by dissolving 5 mg of collagenase in 1 ml of sterile PBS containing 150 mM NaCl and 50 mM NaH2PO4 at pH 7.4. The sham procedure involved percutaneously injecting 15 μl of sterile PBS into the left Achilles tendons. After the procedure, rats were placed under a heat lamp until they awakened.

**Cell proliferation assay.** Immediately after awakening, the rats were placed in their respective cages where they were provided with 0.1% S'-bromo-2'-deoxyuridine (BrDU; Sigma) solution as drinking water to detect cell proliferation in injured tissues. BrDU (C6H12BrN4O4) is a thymidine analog selectively incorporated into DNA at the S phase of the cell cycle that makes it possible to detect DNA synthesis in tissue sections. Animals from each test group were sacrificed at days 0, 1, 3, 7, 14, and 28 after tendon injury.

**Tissue preparation.** Hindlimbs were removed from the animals and killed and were fixed in a dorsiflexion position overnight in 10% formalin at 4°C to label PMNs, ED1⁺, and ED2⁺ MΦs. In another set of experiments, hindlimbs were fixed for 4 days in a zinc solution containing 0.5% ZnCl and 0.5% zinc acetate in Tris-CaCl₂ solution buffer (pH 7.0) for efficient labeling of platelet endothelial cell adhesion molecule-1 (PECAM-1) and BRDU antibodies (1). The Achilles tendons were then excised between the osteotendinous and myotendinous junctions, dehydrated, embedded in paraffin, and sectioned as described by Marsolais et al. (17). Two 5-μm-thick longitudinal sections were collected from two distinct regions in each tendon (peripheral and central) located 0.2 and 0.7 mm from the lateral edge of the tendon, respectively.

**Immunohistochemistry.** PMNs, ED1⁺ and ED2⁺ MΦs, blood vessels, and proliferative cells were labeled by using the following antibodies: anti-PMN (rabbit anti-rat, diluted 1/12,000 in PBS; Accurate Chemical, Westbury, NY), anti-ED1⁺ or anti-ED2⁺ MΦ (mouse anti-rat, diluted 1/200 in PBS; Serotec, Raleigh, NC), anti-PECAM-1 (mouse anti-rat, diluted 1/100 in PBS; BD Pharmingen, San Diego, CA), and anti-BRDU (mouse anti-rat, diluted 1/400 in PBS; Sigma), which reacts specifically with BrDU in nuclei to detect DNA replication in tendon cells. All sections were then washed three times with PBS for 10 min each and incubated with biotinylated anti-rabbit or anti-mouse IgG (diluted 1/200 in PBS; Vector Laboratories, Burlingame, CA) for 1 h. After being rinsed in PBS, the tissue samples were incubated with horseradish peroxidase avidin-D (diluted 1/1,000 in PBS; Vector), washed in PBS, revealed by using a peroxidase diaminobenzidine chromat kit (DAKO, Carpinteria, CA), and mounted on coverslips. The sections were observed by using an inverted microscope equipped with a charge-coupled device camera.

**Positive cells were counted manually, and cell concentrations were calculated on the basis of the section area and thickness. Images of the sections were acquired, and the surface areas occupied by blood vessels in the central and peripheral regions of the Achilles tendon were quantified and expressed as percentages of total areas by using MetaMorph software (Universal Imaging, Downingtown, PA).

**Statistical analysis.** All values are presented as means ± SE. Differences between the concentrations of PMNs, MΦs, and proliferative cells as well as the densities of blood vessels in all groups were assessed by one-way ANOVA. Fisher’s protected least significant difference test was used to compare means when a significant F ratio was obtained. The level of significance was set at P < 0.05.

**RESULTS**

**Sequence of inflammatory cell accumulation in injured tendon.** To examine the sequence and time course of inflammatory cell accumulation following collagenase-induced Achilles tendon injury, we immunolabeled PMNs (Fig. 1, A and B), ED1⁺ MΦs (Fig. 1, C and D), and ED2⁺ MΦs (Fig. 1, E and F) in injured tendons and quantified their numbers at days 0, 1, 3, 7, 14, and 28 posttrauma in nondiabetic, diabetic, and insulin-treated diabetic animals. Percutaneous injection of collagenase in Achilles tendons caused considerable tissue degradation and accumulation of inflammatory cells in all of the groups.

The concentration of PMNs in Achilles tendons from diabetic rats was significantly less (46%) by day 1 compared with nondiabetic animals. ED1⁺ MΦs were the second population of inflammatory cells to invade the Achilles tendon. Their concentration increased progressively and peaked at day 3 postinjury in all groups (Fig. 2B). In diabetic rats, the concentration of ED1⁺ MΦs was reduced by 43 and 35% by days 1 and 3, respectively, compared with nondiabetic rats. Lastly, the concentration of endogenous ED2⁺ MΦs increased slightly and peaked at days 3 and 7 postinjury before returning to normal at day 28 posttrauma (Fig. 2C). The concentration of ED2⁺ MΦs was also reduced by 52% by day 3 postinjury in diabetic compared with nondiabetic animals. The daily injection of regular and ultralent insulin restored the number of inflammatory cells.

**Neovascularization of injured tendon during diabetes.** Because matrix formation and fibroblast proliferation depend on angiogenesis, a process that occurs through the migration, proliferation, and organization of vascular endothelial cells, we next labeled blood vessels (Fig. 1, G and H) and assessed their concentration in injured Achilles tendons. We quantified the density of blood vessels in the paratenon and the core of the tendons at days 0, 3, 7, 14, and 28 posttrauma (Fig. 3). Uninjured Achilles tendons are poorly vascularized, and blood vessels are highly concentrated in the paratenon, where they constitute 5.5% of the total area. For all groups, angiogenesis
peaked at days 7 and 14 in the paratenon and the core of the tendon, respectively, following tendon injury (Fig. 3). The blood vessel density then gradually decreased and reached control values at day 28 postinjury. In addition, diabetic rats presented a 35 and 29% reduction in the formation of new vessels in the paratenon and the core of the tendon, respectively, at days 3 and 7 posttrauma.

Diabetes modulates cell proliferation. To investigate whether diabetes alters cell proliferation, we quantified the number of proliferative cells in injured anti-BrDU-immunolabeled tendons at days 0, 7, 14, and 28 posttrauma (Fig. 1, I and J). No proliferative cells were detected in the absence of injury (data not shown). The immunohistochemical detection of BrDU-labeled nuclei revealed a marked proliferation of cells in the paratenon at day 7 in all groups (Fig. 4A). However, cell proliferation was retarded in the core of the tendon relative to paratenon, which peaked at day 14 (Fig. 4B), suggesting that healing is an inward-directed process. We also observed that cell proliferation in the tendons of diabetic rats was reduced by 34% in the paratenon at day 7 postinjury compared with nondiabetic rats (Fig. 4A).

DISCUSSION

The primary function of tendons is to transmit tension between muscle and bone. Loss of tendon integrity can lead to major disabilities. Achilles, patellar, extensor carpi radialis brevis (epicondylitis), and rotator cuff (tendonitis of the shoul-
Tendons are the most frequently injured by repetitive tasks and sport activities and present a major medical concern for physicians, physical therapists, and research scientists. For example, textile workers have a 12% prevalence of overuse tendonitis and related disorders (20). The prevalence can rise to 25–50% in those who perform repetitive occupational tasks (16, 28). For unknown reasons, diabetic patients are also more at risk of developing tendonitis. Abnormal organization of collagen networks may explain the greater incidence of tendon injury in these patients (9). Although the role of diabetes in perturbing wound healing has been confirmed in skin, intestine, and other tissues (30), the impact of diabetes has not been extensively assessed and characterized during the initial phase of tendon repair.

The present findings show that the sequence of inflammatory cell invasion was preserved. However, the magnitude of the inflammatory response was reduced in diabetic rats following tendon injury. The reduction of PMNs and ED1+ and ED2+ MΦs ranged between 35 and 46% in diabetic rats compared with nondiabetic animals. The impact of these reductions on inflammatory cell numbers remains controversial. For example, PMNs can secrete interleukin-8 and vascular endothelial growth factor, which promote tissue repair. However, they also release a battery of proteases, which can induce substantial tissue damage (21, 27). Recent studies showed that PMN-depleted mice exhibit significantly accelerated reepithelialization, but there is no effect on wound strength, collagen content, or MΦ infiltration (3). On the other hand, the role of MΦs in wound healing appears to be less conflicting than that of PMNs. MΦs can secrete a myriad of cytokines that regulate chemotaxis, angiogenesis (25, 31), fibroblast proliferation (4), scar formation (29), extracellular matrix (ECM) synthesis, and remodeling (10), whereas MΦ-depleted animals present defective wound repair (12). Thus the physiological significance of this global reduction in cell accumulation remains to be established in injured Achilles tendons from diabetic rats. However, our results suggest that a normal, transitory inflammatory response is essential for the normal progression of tissue healing.

The observation that cell proliferation/migration increased and occurred earlier in the paratenon than in the core of the Achilles tendon was an unexpected finding. However, in hindsight, it supports previous results indicating that most blood vessels are located in the paratenon (18). This very ordered sequence of healing from the wound margin inward is not unique to tendon repair (Fig. 5). For example, skeletal muscle regeneration is characterized by MΦ migration through a persisting basal lamina and coincides with a dramatic proliferation of satellite cells that establish a new population of...
Our observations that diabetic rats have impaired neovascularization and reduced MΦ accumulation are also in agreement with another study on skin injury that reported a reduction in angiogenesis and the formation of granulation tissue in diabetic db/db mice compared with normal littermates (5). In addition to their potential role as growth factor-secreting cells, MΦs can potentiate angiogenic activity by releasing matrix metalloproteases and plasmin that in turn release proangiogenic and antiangiogenic factors sequestered within the ECM. Interestingly, the level of urokinase plasminogen activator decreases in the diabetic state, which prevents the degradation of ECM and the outgrowth of capillaries from preexisting capillaries (34). Moldovan et al. (23) also suggest that MΦs may play a role in revascularization by “digging” channels that become invested with endothelial cells to form capillaries. Consequently, it is plausible that ED1+ MΦs play an early role in initiating angiogenesis in Achilles tendon through the release of growth factors and metalloproteases, and the reduction of their concentration in diabetic rats could impair angiogenesis and tendon healing.

The present findings also showed that tendon healing in diabetic rats was associated with a delay in the proliferative phase in the paratenon at day 7 posttrauma. Other in vitro and in vivo studies have clearly demonstrated that cell proliferation is perturbed during diabetes. For example, genetically diabetic mice present a major reduction of cell proliferation and an aberrant control of apoptotic cell death at day 7 during skin repair (2). Furthermore, the isolation of fibroblasts from chronic nonhealing ulcers in diabetic patients exhibit a reduced proliferative capacity in culture (15). These fibroblasts have abnormal morphological features such as multiple lamellar and vesicular bodies as well as no microtubular structures and enlarged endoplasmic reticuli. The lack of microtubules is noteworthy given the well-established role of microtubules in the regulation of cell migration and division.

Together, the results of the present investigation support the hypothesis that the accumulation of inflammatory cells, angio-

Fig. 4. Quantification of proliferative cells in the paratenon (A) and the endotenon (B) from control, nondiabetic, diabetic, and insulin-treated diabetic animals. *Significantly different from nondiabetic animals. Data are means ± SE from 5–6 experiments. P < 0.05.

myoblasts and eventually myotubes. MΦs phagocytose necrotic muscle fibers and migrate centripetally followed by a new network of capillaries (26). Although the source of the cells that participate in tendon healing has yet to be defined, local release of growth factors may activate inflammatory cells and fibroblasts in the tendon sheath and initiate the healing process in the periphery of the Achilles tendon.

Previous results showed that T lymphocytes and MΦs accumulate mainly in the tendon sheath and epitenon 3 days after crush injuries (33). The infiltration of inflammatory cells into the synovial sheath and epitenon would precede an increase in fibronectin production by tendon cells. These observations are consistent with the fact that the paratenon and tendon sheath are highly vascularized and that blood constituents as well as provisional ECM rapidly appear in the peripheral regions of tendons. MΦs, fibroblasts, and blood vessels then move into the wound space at the same time. MΦs provide a continuing source of growth factors necessary to stimulate fibroplasia and angiogenesis. Although angiogenesis is a complex process that relies on ECM, cell migration, and mitogenic stimulation of endothelial cells, the induction of angiogenesis is possibly initiated sequentially by basic growth factors and vascular endothelial growth factor. Basic fibroblast growth factor would set the stage for angiogenesis during the first 3 days of wound repair, whereas vascular endothelial growth factor is critical for angiogenesis during the formation of granulation tissue on days 4–7 (25). Current results also support the observation that basic fibroblast growth factor-2-induced angiogenesis is inhibited by 75% in nonobese diabetic mice compared with control mice.

Fig. 5. Schematic representation of cell appearance and distribution in the paratenon and the core of the tendon. Leukocytes, blood vessels, and proliferative cells appear earlier and in greater numbers in the paratenon than in the core following tendon injury.
genesis, and cell proliferation are impaired in tendon healing in the diabetic state. The administration of insulin restored a normal inflammatory response, neovascularization, and cell proliferation during tendon repair. Since tendons possess a limited repair potential, the perturbation of key biological processes in diabetic rats may be of major importance in tendon healing.

ACKNOWLEDGMENTS

We thank David Marsolais and Charles Godbout for reading the manuscript and for valuable comments.

GRANTS

We gratefully acknowledge financial support from the Natural Sciences and Engineering Research Council of Canada and the Fonds de la Recherche en Santé du Québec.

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