Bone 43 (2008) 832–839

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Molecular basis for affected cartilage formation and bone union in fracture healing of the streptozotocin-induced diabetic rat

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ARTICLE INFO

Article history:
Received 25 May 2008
Revised 21 July 2008
Accepted 25 July 2008
Available online 7 August 2008

Edited by: Thomas Einhorn

Keywords:
Diabetes
Fracture healing
Cartilage
Bone union
Molecular basis

ABSTRACT

Most studies have focused on the association between diabetes mellitus (DM) and impaired osseous healing, but there is also evidence that diabetes impairs cartilage formation during fracture healing. To investigate the molecular mechanisms by which diabetes affects endochondral ossification, experiments were performed in a model of rat closed fracture healing complicated with diabetes. Diabetic rats were created by a single intravenous injection of streptozotocin (STZ), while controls were treated with vehicle alone. Fractures were made 2 weeks after STZ injection. Animals were killed at 4, 7, 10, 14, 21, 28 and 42 days following fracture, and samples were subject to radiographic, histological and molecular analyses. In the DM group, a significantly smaller cartilaginous callus was formed compared with controls throughout healing, with the cartilage area being reduced rapidly after day 14. When the bone union rate was evaluated radiographically on day 28, DM calluses exhibited a lower rate than controls. However, when evaluated on day 42, both groups showed an equivalent union rate. Cellular proliferation of chondroprogenitor cells and proliferating chondrocytes in soft calluses of the DM group was significantly reduced during early stages of healing (days 4 and 7), but no longer reduced thereafter. Moreover, expression levels of collagen type II, type X and osteopontin (OPN) were constantly low in the DM group. These results show the molecular basis for diminished cartilage formation and delayed union in fracture healing of the STZ-induced diabetic rats.

Introduction

The association between diabetes mellitus (DM) and impaired osseous healing has been documented in clinical and experimental settings. Several clinical series have noted that the healing time for diabetic patients is approximately twice as long as that of non-diabetic patients [1,2]. In addition, diabetic patients undergoing arthrodesis had a significantly increased incidence of delayed union, non-union and pseudoarthrosis [3–5]. Chemically-induced and spontaneously developed diabetic animal models have demonstrated impaired fracture healing. In the various models of fracture healing, diabetes has led to reduced biomechanical properties of healing bones, reduced proliferation in the early callus, and reduced collagen synthesis and content compared to non-diabetic control animals [6–13].

One of the best-characterized models to study the impact of diabetes on bone in rats and mice is the induction of type 1 diabetes by streptozotocin (STZ). Treatment with STZ stimulates a host response that leads to destruction of pancreatic β cells, hypoinsulinemia and hyperglycemia, with many features similar to type 1 diabetes in humans [14–16]. Studies using this animal model show that there is a significant decrease in BMD, BMC and serum osteocalcin levels in the STZ-induced diabetic animals [17,18]. Moreover, there is a 20% decrease in biomechanical strength in both femurs and tibias [18,19]. BB rats, which are spontaneously diabetic, show similar reductions in BMD, serum osteocalcin levels and mechanical properties [20,21]. Long bone fractures of spontaneously diabetic or STZ-induced diabetic animals exhibit smaller calluses with decreased bone formation, proliferation and differentiation of osteoblastic cells and a 2-fold reduction in mechanical strength compared to matched controls [6,8,22,23].

Although most of the studies have focused on bone, there is also evidence that diabetes impairs cartilage formation (chondrogenesis) during fracture healing through decreased chondrocyte differentiation and proliferation [6,13,22,23]. Chondrogenesis, an essential component of endochondral ossification in long bones, is a key component of fracture healing. Growth factors and cytokines that are produced by the inflammatory response to skeletal injury support

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doi:10.1016/j.bone.2008.07.246

chemotaxis of immature mesenchymal cells to the site of fracture and promote their differentiation into chondrocytes. A well-organized development and maturation process leads to the formation of mineralized cartilage, which is subsequently replaced by bone [24,25]. It is therefore conceivable that diabetes impairs fracture healing by affecting not only bone (intramembranous) formation, but also cartilage (endochondral) development. However, the molecular mechanisms by which diabetes affects endochondral ossification in fracture healing have not been fully determined.

In the present study, a closed femoral shaft fracture was created in STZ-induced diabetic rats. We then performed detailed histological and molecular analyses, focusing on the spatial and temporal expression of cartilage-related collagen and non-collagen genes, to determine the impact of diabetes on fracture healing, particularly on endochondral ossification at molecular levels.

Materials and methods

Animals and materials

Two-month-old male Sprague–Dawley rats (10–12 weeks old; 300–400 g) were divided into two groups (control, $n=64$; diabetic, $n=62$). As a model for impaired fracture-repairing ability, diabetic rats were created by a single intravenous injection of 40 mg/kg STZ (Roche Molecular Biochemicals, Indianapolis, IN) in 0.1 M citrate buffer (pH 4.9). Rats with blood glucose levels over 300 mg/dl at 1 week after injection were used for experiments [7,13], and fractures were made 2 weeks after STZ injection. During experiments, the blood glucose levels were checked twice a week, and animals were excluded from the study if the blood glucose levels were less than 300 mg/dl in two consecutive measurements.

On days 4, 7, 10, 14, 21 and 28 following surgery, eight rats from each group were killed and the samples harvested in preparation for molecular ($n=4$) and histological ($n=4$) analyses. Day 42 samples (control, $n=12$; diabetic, $n=11$) were used only for radiographic analysis.

Fracture model

A standard, closed, mid-diaphyseal fracture was produced in the right femur of each rat according to Dr. Einhorn's fracture model [26]. Following anesthesia, a Kirschner wire (1.1 mm in diameter) was introduced into the medullary canal of the right femur, and a mid-diaphyseal fracture was created with an apparatus composed of a blunt guillotine driven by a dropped weight. These experimental procedures were approved by the Animal Care and Use Committee of Chiba University, Japan.

Radiographic analysis

When animals were sacrificed, radiographs were taken 7, 14, 28 and 42 days after the fracture. To judge bone union, eleven to twelve calluses from the control and DM groups were evaluated radiographically on days 28 and 42 post-fracture. On radiographic evaluation, four cortices (two on the antero-posterior and two on the lateral radiograph) on each callus were evaluated by at least two different authors (all are Orthopaedic surgeons), and a fracture callus was defined as a bony union when three of four cortices were bridged.

Tissue preparation

Four rats from each group were killed by intracardiac infusion of 4% paraformaldehyde under sodium pentobarbital anesthesia on days 4, 7, 10, 14, 21 and 28 (total 24 rats from each group) after production of fractures. Fractured femurs were removed with the surrounding tissues and fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4 °C for 24 h. Tissues were decalcified at room temperature with 20% EDTA, 0.05 M Tris–HCl (pH 7.4), bisected sagittally in the median plane, and embedded in paraffin. Six-micrometer mid-sagittal sections were mounted on silane-coated slides.

Measurement of cartilage area

To visualize cartilage matrices, sagittal sections were stained with toluidine blue (pH 4.1), and areas showing metachromasia were identified as cartilage. Each callus was photographed using a standard light microscope. The cartilage area showing metachromasia was analyzed using NIH image (http://rsb.info.nih.gov/nih-image/).

Analysis of cellular proliferation in the soft callus

The fracture callus can be histologically divided into 'soft' and 'hard' calluses. Soft calluses consist of fibrous and cartilage tissues which are formed by endochondral ossification, while hard calluses consist of bone tissues which are formed by intramembranous ossification.

Sections were immunostained with a monoclonal antibody against proliferating cell nuclear antigen (PCNA) (PC-10; DAKO Japan, Kyoto, Japan) to evaluate cell proliferation. Immunochemical staining was performed as previously described [24,25,27,28]. Signals were detected using diaminobenzidine (DAB) followed by counterstaining with methylgreen. Four regions in the soft callus including cartilage or non-cartilaginous tissues were analyzed in each specimen, and the number of PCNA-positive cells was counted on days 4, 7 and 14 after fracture. Then the ratio of PCNA-positive cells to total cells was calculated and expressed as a percentage. The measurements were performed at least three times for one location of each specimen, and the average measurement obtained was defined as the PCNA score [24,25,27–29].

Preparation of probes

The following cDNA clones were used as hybridization probes in this study: mouse pro-α1 (II) collagen (COL2A1) cDNA containing a 0.64-kb fragment, mouse pro-α1(X) collagen (COL10A1) cDNA containing a 0.60-kb fragment and mouse osteopontin (OPN) cDNA containing a 1.2-kb fragment (a gift from Dr. S. Nomura, Osaka University, Japan). Specificity of these probes was confirmed previously [24,25].

RNA extraction and Northern blot analysis

For RNA extraction, rats were killed as described above on days 4, 7, 14, 21 and 28 post-operatively (total 20 rats from each group) and the fractured femurs were harvested. Tissues were frozen immediately in liquid nitrogen and stored at −80 °C until RNA isolation was performed. Total cellular RNA was extracted and mixed from four calluses for each group at different time points using TRIzol (Gibco BRL, Rockville, MD) according to the manufacturer's instructions. Twenty micrograms of total RNA from each daily sample was subjected to 1% agarose gel electrophoresis and transferred to a nylon membrane (Hybond-XL; Amersham Pharmacia Biotech, Buckinghamshire, UK). cDNA probes were labeled with ^{32}P using a random priming method. Northern blot analysis was carried out as previously described [24,25,27,28]. The density of each band on the autoradiogram was estimated by an image analyzer (Image Gauge software, version 3.1; FUJIFILM, Tokyo, Japan).

In situ hybridization

To compare distribution of cells expressing OPN mRNA in the fracture callus between groups, sections were hybridized with probes for OPN. Digoxigenin (DIG)-11-uridine 5-triphosphate-labeled single