GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Observed impacts of insulin therapy on callus cell transforming growth factor-beta 1 expression in diabetic rats

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Genet. Mol. Res. 14 (2): 5076-5084 (2015)
Received August 7, 2014
Accepted November 4, 2014
Published May 12, 2015
DOI http://dx.doi.org/10.4238/2015.May.12.10

ABSTRACT. The expression of transforming growth factor-beta 1 (TGF-β1) inside the callus cells of diabetic rats and the impact of insulin therapy on its expression and biomechanics was investigated. The rats were randomly divided as follows: an insulin therapy group (IT), a diabetic model group (DM), and a non-diabetic control group (NC). Bone specimens from each group were extracted at different times for immunohistochemical observation of the expression of TGF-β1. Concurrently, the destruction torque and torsional stiffness were detected at different times. One to four weeks after fracture, TGF-β1 was widely expressed in fractured callus cells and periosteal proliferating cells, while the expression inside diabetic cells was significantly reduced. The expression of TGF-β1 decreased over the first 68 weeks, and the mature bone cells never expressed TGF-β1. The destruction torque (Nm) detected in the 6th week revealed that there was a statistically significant difference between the DM, NC, and IT groups ($P < 0.01$). In conclusion, TGF-β1 expression was significantly

reduced inside the callus cells of diabetic rats. Insulin therapy increased TGF-β1 expression inside the callus cells of diabetic rats and improved the biomechanical characteristics of the callus.

Key words: Diabetes; Fracture; TGF-β1; Insulin

INTRODUCTION

Diabetes is a serious chronic disease that has become the third most common chronic non-communicable disease after cardiovascular disease and cancer, posing a serious and increasing public health risk. The main effects of diabetes on the locomotor system are reflected in a reduction of bone formation leading to osteoporosis, a decrease of bone mechanical strength causing fractures and adverse effects on fracture healing, but currently little is known about the mechanisms involved. Studies on the effects of diabetes on fracture healing are limited; they directly focus on a particular factor of fracture healing, or are observational studies on individual diabetic fracture cases.

Diabetes might affect fracture healing through its effects on cells and cytokines. Krakauer et al. (1995) performed a 12-year prospective clinical study in diabetic patients, the results of which showed that the diabetic population exhibit significantly less bone formation than the normal population. During the process of fracture healing, the relationships between cells and cytokines are complex, and thus the impact of diabetes on cells and cytokines is complex. Diabetes might reduce the levels of growth factors at the fracture site, thus affecting the fracture healing process (Scherpereel and Tavernier, 2001), and it might inhibit the release of growth factors and the expression of a series of genes originating from normal inflammatory cells.

Other studies have shown that insulin intervention can improve the fracture healing process of diabetic patients. *In vitro* studies have found that under an environment of insulin deficiency, cartilage formation is delayed and ossification/calcification is reduced, indicating that insulin might act as a circulating factor, thus playing an important role in the formation of connective tissues. Insulin could improve the fracture healing process in diabetic patients, but the mechanism of this adjustment is not very clear yet. Fracture healing is affected by the interaction of multiple factors such as hormones, cells, extracellular matrix, cell growth factors, and a variety of bone growth factors that are involved in regulating cell proliferation, differentiation, and synthesis of the extracellular matrix. To date, multiple healing-promotion growth factors have been found at the fracture site such as transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), and vascular endothelial growth factor (VEGF). The changes in the expression of various growth factors at the fracture sites of diabetic patients and the impact of insulin therapy on their expression have not been reported. The purpose of this study was to observe the expression pattern of TGF-β1 inside the callus cells of diabetic rats, and assess the impact of insulin therapy on its expression and callus biomechanical characteristics.

MATERIAL AND METHODS

Preparation of experimental animal models

Two kinds of disease models were established in this experiment using the same

animal. Preliminary experiments tested the New Zealand white rabbit and rat. The diabetic rabbit model was successfully established; however, the survival time was too short to reach the experimental requirements, and the rabbit animal model was abandoned. Ultimately, the rat was chosen to create the animal model for our experiment because it was easier to establish a rat model of diabetes, and the rat is the most popular animal for studies of fracture healing with the femur, tibia, fibula, ulna, and radius all suitable for fracture modeling (Thaller et al., 1993; Hietaniemi et al., 1995; Lynch et al., 1999). Male Wistar rats (N = 96, provided by the Experimental Animal Laboratory, Shandong Lukang Pharmaceutical Co., Ltd.), weighing 280-320 g, were randomly divided into three groups. The diabetic model group (DM): After 12 h of fasting, 32 rats were intraperitoneally injected 5% alloxan solution (200 mg/kg body weight; Sigma, USA). The blood glucose was tested 48 h later and rats with blood glucose consistently greater than 16.7 mM were selected for the DM, based on the model standard of diabetic rat, which was 11.1-16.7 mM, under fasting or non-fasting conditions (Lynch et al., 1999). The insulin therapy group (IT): Diabetic rats (N = 32), in which blood sugar was strictly controlled, were administrated insulin therapy, Novolin 30R (40 IU/mL, Novo Nordisk, 4-6 $U \cdot kg^{-1} \cdot day^{-1}$), subcutaneously injected between 5 and 6 pm daily, and the fasting blood glucose was tested on the following day; blood glucose was controlled between 4-9 mM. The non-diabetic control group (NC): Rats (N = 32) were injected with normal saline as the control. The fracture open modeling was referenced to Phieffer et al. (2000), Waters et al. (2000), and Lee et al. (2000): under sterile conditions, a consistent bone defect was surgically performed using either a wire saw, an oscillating saw, or a bone-biting clamp, or the defected bone was not completely broken, or the double bones were sawn through on just one non-load bearing bone or one load-bearing bone. There was an additional report of a non-fixed closure fracture (Bourque et al., 1992). The fractures produced by a wire saw or an oscillating saw exhibited a neat section and, if no internal fixation is performed, it would be easy for the sections to slide and be displaced. During our experiment, the sharp-nose rongeur was used to perform the surgery on 1/3rd of the upper tibia. The tibia was bitten off with small bone teeth on the proximal and distal bone ends; the bone teeth were staggered to each other, thus increasing the stability of the fracture. Moreover, with the support of fibula, no other internal or external fixation measures were needed, and the animals were able to perform normal activities after surgery consistent with the human fracture process. After successfully establishing the diabetes model, the rats were subjected to right tibia fracture, which was manually performed under sterile surgical conditions. On the 1st, 2nd, 4th, 6th, and 8th weeks after surgery, the rats were killed to harvest the fractured segment, which was fixed in 10% formalin for 12-24 h, the callus was removed for pathological sectioning, and immunohistochemical staining was performed to observe the TGF-β1 expression. During the 6th and 8th postoperative weeks, 6 rats of each group were killed for detection of destruction torque and torsional stiffness of the bilateral tibia.

Experimental methods

Production of paraffin

The callus was placed in 10% formaldehyde solution for fixation and paraffin-embed-

ment, with a slice thickness of 5 μm.

Immunohistochemical detection

1) TGF-β1 monoclonal antibody was used as the primary antibody (Zhonghao Biopcr Co. Ltd., Beijing), and the sections were stained using the streptavidin-biotin complex (SABC) method, followed by 3,3'-diaminobenzidine (DAB) (Shanghai Gaochuang Chemical Technology Co., Ltd.) staining; the slice was then processed with polylysine and placed into a 60°C oven so that the slices would adhere tightly.

2) Dewaxing paraffin sections to distilled water.

3) The endogenous enzymes were removed: the slice was placed into a 3% aqueous solution of hydrogen peroxide for 20 min, washed with phosphate-buffered saline (PBS); pH 7.2-7.6 for 5 min, repeated 3 times.

4) Digestion with trypsin to expose the antigen: 0.1% trypsin was added, and the mixture incubated at 37°C for 15 min, followed by washing for 5 min in PBS, repeated 3 times.

5) Bovine serum albumin (5%) closure solution was added drop wise, and the mixture incubated at room temperature for 20 min. The excess liquid was discarded without washing.

6) The 1:150 diluted primary antibody was added drop wise, and the mixture was incubated overnight at 4°C, before washing in PBS for 5 min, repeated 3 times.

7) The appropriately diluted biotinylated secondary antibody was added drop wise, and kept in the wet box for 20 min, followed by rinsing with PBS for 5 min, repeated 3 times.

8) The SABC was added drop wise, and kept in the wet box for 20 min, followed by rinsing with PBS for 5 min, repeated 4 times.

9) Addition of chromogenic substrate: DAB-H_2O_2 staining solution was added and kept at room temperature for 1 min. The staining time was controlled using a microscope, and rinsing in water was used to terminate the reaction.

10) Hematoxylin (Shang Hai Solarbio Biological Technology Co., Ltd.) was used to restain the nucleus, and the dehydrated, hyalinized, and closed slice was examined under a light microscope.

Results judgment: A brown or brownish-black stained cytoplasm with a blue nucleus represents a positive result.

Biomechanical testing

The animals were randomly killed during the 6th and 8th week post-fracture-operation, with 6 rats from each group killed at each time point. The complete tibial shaft was removed, and concurrently, the contralateral tibia was removed to calculate the parameters' ratio. The soft tissues were cleared, and the bone was wrapped in gauze, soaked in saline, and stored at -20°C in a double-plastic sealed bag for future whole batch testing. When used for detection, the specimen was thawed and placed in saline for 30 min, and then the two ends of each specimen were embedded with self-curing denture powder, followed by fixation on the mechanical testing machine (858Mini Bionix, Biomechanics Laboratory, Southern Medical University, USA). The direction and location of the tibial specimens were maintained relatively consistently during the determination to reduce system error.

Test items:

1) Torsional stiffness test: torsional loading was added onto the fracture at a rate of 15 mm/min; the torsional stiffness (N.mm/degree) per cm was automatically calculated using computer software according to the load angle-displacement curve.

2) Torsional strength test: torsional force was loaded onto the fracture at a rate of 4°/min; the torsional strength (N.mm) was automatically calculated using computer software according to the load angle-displacement curve.

3) The corresponding non-surgical side specimen was tested simultaneously to calculate the ratio of mechanical parameters of each pair of samples.

Statistical methods

The SPSS12.0 statistical software was used for the statistical analysis, with the experimental data reported as means ± SD. The Dunnett *t*-test was used to measure the significance of multiple-sample means.

RESULTS

Immunohistochemical results

A positive TGF-β1 expression was indicated by brown staining.

1) During the 1st week post-fracture, immunohistochemical staining of the callus tissue for TGF-β1 (Figure 1A, B, C) showed that the cytoplasm of the fractured periosteal mesenchymal cells, chondroblasts, and cartilage cells exhibited TGF-β1 expression. Among these, the expression of TGF-β1 was stronger in the cartilage cells, while its expression in the DM was weaker than that in the other two groups.

2) During the 2nd week post-fracture, immunohistochemical staining of the callus tissue for TGF-β1 (Figure 1D, E, F) showed that the cytoplasm of the fractured periosteal mesenchymal cells, proliferated cartilage cells, and active chondroblasts of the IT exhibited TGF-β1 expression. The expression of TGF-β1 was stronger in the cartilage cells and chondroblasts of the NC, while its expression in the DM was weaker than that in the other two groups. Furthermore, the cartilage cells in the functional areas of each group weakly expressed or did not express TGF-β1.

3) During the 4th week post-fracture, immunohistochemical staining of the callus tissue for TGF-β1 (Figure 1G, H, I) showed that the cytoplasm of the chondroblasts and immature osteocytes exhibited TGF-β1 expression, while its expression in the DM was weaker than that in the other two groups.

4) During the 6th week after fracture, immunohistochemical staining of callus tissue for TGF-β1 (Figure 1J, K, L) showed that the expression of TGF-β1 began to decrease in the cytoplasm of the osteocytes, while the mature osteocytes did not express TGF-β1. Furthermore, the expression of TGF-β1 in the chondroblasts and osteocytes of the DM also began to decrease.

5) During the 8th week after fracture, immunohistochemical staining of callus tissue for TGF-β1 (Figure 1M, N, O) showed that the callus tissue of each group formed layers, the osteocytes were mature, and the expression of TGF-β1 in the cytoplasm was weak or was non-expressed.

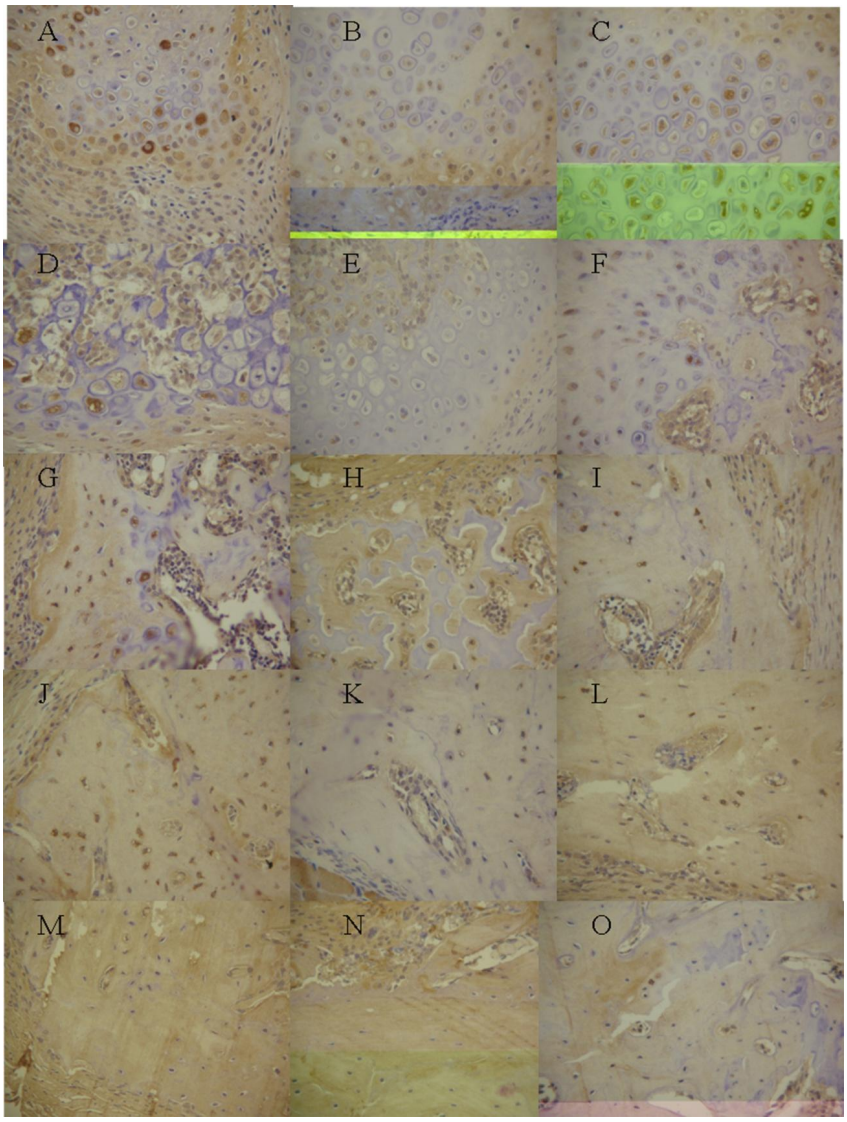

Figure 1. Immunohistochemical sections of callus tissue for TGF-β1 on the 1st week of fracture: **A.** Insulin therapy group (IT); **B.** diabetic model group (DM); **C.** IT. On the 2nd week of fracture: **D.** IT; **E.** DM; **F.** IT. On the 4th week of fracture: **G.** IT; **H.** DM; **I.** IT. On the 6th week of fracture: **J.** IT; **K.** DM; **L.** IT. On the 8th week of fracture: **M.** IT; **N.** DM; **O.** IT.

Biomechanical test results

The destruction torque and torsional stiffness of the 3 groups during the 6th and 8th weeks post-surgery are shown in Table 1. The results showed that the biomechanical characteristics of the DM decreased, while insulin therapy improved the damaged biomechanical characteristics.

Table 1. After the first 6 weeks, three groups of specimens destroyed torque and torsional stiffness.

Group	Destruction torque (Nm)	Percentage of destruction torque	Torsional stiffness (Nm/rad)	Percentage of torsional stiffness
6th week				
IT	360 ± 77	54 ± 13	19,800 ± 6150	80 ± 34
DM	178 ± 84[a,b]	25 ± 10[a,b]	9,420 ± 3760[a,b]	29 ± 18[a,b]
NC	414 ± 50	69 ± 17	20,900 ± 3940	72 ± 26
6th week				
IT	401 ± 80	65 ± 28	23,100 ± 8880	71 ± 33
DM	250 ± 108[a,b]	42 ± 15[c]	10,700 ± 4120[a,b]	36 ± 19[c]
NC	500 ± 54	81 ± 21	26,500 ± 4180	89 ± 31

At the same time point, the diabetic model group (DM) compared to the non-diabetic control group (NC), [a]$P < 0.01$, compared with the insulin therapy group (IT), [b]$P < 0.01$, and compared with the NC, [c]$P < 0.01$.

DISCUSSION

Fracture healing is a complex process of bone regeneration, and there are many factors involved in the regulation of this biological process. Currently, the important cytokines involved in fracture healing include BMP, TGF-β, FGF, PDGF, IGF, and VEGF. Although these growth factors are minimal in tissues or plasma, they play a crucial role in regulating cell division, matrix synthesis, and tissue differentiation. TGF-β is a family of polypeptides with multiple functions that is widely present in normal animal tissue cells and transformants, among which the bone tissues and platelets exhibit the most abundant levels. The family consists of at least five different types of TGF-β molecules. TGF-β (Sporn and Roborts, 1992) can promote cell proliferation and differentiation, extracellular matrix synthesis, and it is also an autocrine- or paracrine-regulatory factor in a variety of immune cells. TGF-β can adjust the proliferation of chondrocytes, osteoblasts, and osteoclasts, as well as the expression of different cellular phenotypes and can regulate the expression of insulin-like growth factor-binding protein 4 (IGFBP-4) and pregnancy-associated plasma protein A (PAPP-A) in cultured periosteal explants (Gonzalez et al., 2010). *In vitro* studies (Joyce et al., 1990) have shown that during the process of endochondral ossification, the chondrocytes and osteoblasts can synthesize TGF-β, which then largely accumulates inside the extracellular matrix. The highest content of TGF-β appears in the *in vivo* extraosteocyte matrix. TGF-β also plays an important role in the synthesis of extracellular matrix. During the fracture-healing process, the local levels of TGF-β1 significantly increase inside the bones (Li et al., 2012) and the hematoma; increasing serum TGF-β1 levels of fracture patients demonstrate their importance, especially after the fracture-healing process (Sarahrudi et al., 2011). Studies have found that TGF-β can not only stimulate the cranioaural cells to synthesize collagen, but also induce it to produce a variety of matrix proteins such as fibronectin, osteonectin, and matrix glycoproteins. The implantation of TGF-β and demineralized bone matrix complex into the fractured location could improve and accelerate bone repair (Servin-Trujillo et al., 2011). TGF-β can enhance BMP-induced osteoblast differentiation, which might be beneficial to fracture healing (de Gorter et al., 2011).

Foreign and domestic scholars observed that diabetes limited cell proliferation during the fracture healing process in experimental animal models. Kayal et al. (2007) considered that the characteristic of reduced fracture-healing ability in diabetic patients was because of increased cartilage resorption rate leading to a lower formation of callus, thus decreasing bone formation and mechanical strength. Based on Gooch et al. (2000) study, the maturation of chondrocytes in diabetic animals was delayed and increased excessively histologically. Gen-

erally, the average time of fracture healing in the DM was 1 week or so later that in the NC (Lammens et al., 1997; Funk et al., 2000). This effect might be realized through inhibition of the expression of cytokines at the fracture site. Some scholars have also inferred that insulin might affect the expression of key factors during fracture healing. In this experiment, the TGF-β1 monoclonal antibody was used to detect the expression of TGF-β1 in the proliferating cells at the fracture site. The expression levels are represented by the shades of staining, and by controlling the blood sugar with insulin, the impact of cell proliferation on TGF-β1 expression was observed. The expression of TGF-β1 occurred through the complete process of callus cell maturation, exhibiting different expression levels at different stages. During the 1st to 4th week, a strong expression was exhibited in the periosteal proliferating cells, active chondrocytes, and osteoblasts, while from the 6th to 8th week, the knitting callus developed into the callus layers, the osteocytes in the fracture site gradually matured, TGF-β1 expression began to decline, and the fully mature osteocytes no longer expressed TGF-β1. During the maturation of cartilage callus, the hypertrophic chondrocytes either expressed little or no TGF-β1, and at the same time, the osteoblasts began to aggregate. The TGF-β1 expression in the DM was weaker than that in the IT and NC, indicating that insulin could increase the expression of TGF-β1 inside the proliferated cells at the fracture site. Values from the 6th and 8th weeks for torsional strength and torsional stiffness from the 3 groups revealed that the biomechanical characteristics of the DM decreased, while insulin therapy improved the injured biomechanical characteristics.

The reduction in TGF-β1 expression in the callus proliferated cells in diabetic rats is one of the important causes of the impact of diabetes on fracture healing, which lays a foundation for the further understanding of the mechanisms of diabetes-impacted fracture healing. Fracture healing is a complex process of bone regeneration involving many factors with mutual and complex influences. Insulin therapy could improve fracture healing by increasing TGF-β1 expression by the proliferated cells in the fracture site. Whether this is a direct effect of insulin or is indirectly achieved through the effects of other cytokines requires further investigation.

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