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Expression of TGF-β in Fractures Fixed by Nitinol Swan-like Memory Compressive Connectors

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In this article, the effect of internal fixation of a Nitinol swan-like memory compressive connector (SMC) on the temporal expression of transforming growth factor-β (TGF-β) at fracture sites is evaluated. Specimens were collected from 35 New Zealand rabbits modeled for bilateral humeral fracture fixed with either SMC or stainless dynamic compression plate (DCP). Five rabbits each were killed at day 1, 3, 7, 14, 21, 28, and 56. The local positive staining potency, positive area ratio, and positive index of TGF-β were measured using an immunohistochemistry approach (EnVision) in combination with a computerized image analysis system. TGF-β staining was seen in mesenchymal cells, osteoblasts, chondrocytes, and in the extracellular matrix of fractures fixed in both the SMC and the DCP samples without a significant difference in staining at both the early stages (days 1 and 3) and day 56. A higher TGF-β content was observed in the fractures fixed with SMC when compared to that of DCP from day 7 to 28. As a conclusion, TGF-β is highly expressed in fractures fixed with SMC during chondrogenesis stage and entochondrostosis stage. Finally, the mechanism of how SMC promoting synthesis and secretion of TGF-β in the process of fracture healing has been discussed.

Keywords fracture healing, nitinol, swan-like memory compressive connector, transforming growth factor-β

1. Introduction

The Nitinol is an alloy of nickel and titanium that belongs to a class of materials called shape memory alloys. It is flexible at below 10 °C and regains rigidity and its original shape at above 30 °C. Nitinol, with the effect of shape memory and its superiority in wear-resisting and corrosion-resisting, has been extensively applied in the medical field and is considered to be a kind of rare ''bio-memory material.'' The Chinese scholar, Zhang Chuncai, invented the Nitinol swan-like memory compressive connector (SMC) (Ref 1) (Chinese Patent No. ZL99113873.2) to treat fractures in 1986. By taking advantage of the ''thermo-elastic martensite-type'' characteristic of Nitinol, the SMC forms the relatively ideal local mechanical environment necessary for the bio-reaction of the fracture union at the fracture site of the long bone. In addition, the SMC enables persistent and initial axial pressure to be exerted on the fracture end, thereby maintaining a long-term and appropriate initial memory load at the fracture end and preventing the occurrence of osteoporosis and re-fracture (Ref 2, 3).

Studies both in vivo and in vitro have confirmed that transforming growth factor-β (TGF-β) (Ref 4) is an important stimulator for bone growth, particularly in fracture repair (Ref 5). TGF-β does this by adjusting the proliferation and differentiation of mesenchymal cells, promoting proliferation of osteoblasts and chondrocytes, stimulating synthesis of collagen, and inducing intramembranous ossification and entochondrostosis (Ref 6).

The purpose of this study is to explore the temporal TGF-β expression pattern in fractures using an immunohistochemistry approach, and likewise investigate the effect of constant compressive stress on TGF-β expression. The mechanism of how the Nitinol swan-like memory compressive connector (SMC) promotes the fracture healing is also investigated. All above fractures were internally fixed with SMC or stainless dynamic compression plate (DCP) in rabbits.

2. Materials and Methods

2.1 Internal Fixation Devices

The Nitinol swan-like memory compressive connector (SMC): The SMC was invented by Zhang Chuncai from the Department of Orthopedics, Changhai Hospital of Second Military Medical University in Shanghai, China, and produced by Ximai Biological Medical Equipment Corporation (Chinese registration number of the medical equipment: 2002, No. 3040024), complying with the national standards. The SMC (containing nickel and titanium with atomic ratio of 1:1) is made with three parts (Ref 1, 2): connective part (one swan-body), compressive part (two swan-necks), and bone-holding part (four swan-wings), 30 mm long, with an inner diameter of

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7 mm at the proximal end and an inner diameter of 5 mm at the distal end. The heat treatment was single-pass, and the shape rebounding temperature was 33 ± 2 °C. DCP (produced by Shanghai Puwei Medical Devices Factory): stainless material, 4 holes, 30 mm long, 4 mm wide, 1.5 mm thick, and the screw diameter was 2 mm.

2.2 Establishment of Animal Model

35 New Zealand rabbits, male or female, weighing 2.0-2.5 kg, were used. A lateral and longitudinal incision was made on upper arm of forelimb under protection of the cephalic vein and radial nerve exposing bilateral humeral shaft. The bone was cut off at the middle of the humeral shaft using a jigsaw. The SMC was spread in 0-4 °C ice water and arranged according to the standard procedures. The SMC was restored to normal temperature in 40 °C saline, forming a three-dimensional fixation against the fracture (Ref 3). Following this, the incision was closed. The fixation of 4-hole DCP was performed on the opposite limb with the same procedures in the same rabbit. The rabbits were allowed to move freely after the operation without external fixation and were later randomly killed, five each group, at day 1, 3, 7, 14, 21, 28, and 56. The callus, osseous tissues, and surrounding tissues collected from the fractures were prepared as specimens, falling into either the SMC group or the DCP group and treated according to the fixation methods.

2.3 Two-step Immunohistochemistry EnVision

The specimens prepared in paraffin sections were subjected to immunohistochemistry procedures as follows: dewaxing, antigen repair, primary and secondary antibody staining, developing, and bluing (Ref 7, 8).

2.4 Section Observation and Quantitative Analysis

The immunohistochemical sections were observed under the microscope and images collected were analyzed using image analysis software called SPOT. First, selecting a fixed area and defining light or dark brown as positive staining, and calculating the potency value for each section positively stained. Second, measuring the granule area and calculating the ratio of area positively stained versus the area measured (positive area ratio). Third, multiplying the positive staining potency by the positive area ratio to attain the immunohistochemistry positive index (positive index) representing the TGF-β content (Ref 9).

2.5 Statistics

The data were represented in the form of $x \pm s$, and the between-group comparisons were with the paired t test.

3. Results

3.1 Histological Observation

At days 1 and 3 of this study, the hematomas under the periosteum and in the fractured end of both the SMC group and the DCP group were infiltrated by inflammatory cells, meanwhile the extracellular matrix was strongly stained for TGF-β. Four days post-operation, the proliferation and differentiation of the mesenchymal cells and chondrocytes became increased. TGF-β staining was seen in the osteoblasts, particularly in the matrix, with the SMC group having stronger staining than the DCP group (see Fig. 1a,b). The formation of the cartilage callus and trabeculation of bone was seen in the fractured zone of both the groups 14 days after the operation. The TGF-β staining was positive inside and outside of the osteoblasts on the surface of the bone trabecula, but the osteoblasts in the matrix were not stained. The TGF-β staining was strongly positive inside the chondrocytes, and was also positive in the matrix, with the SMC group stronger than the DCP group (see Fig. 1c,d). At days 21 and 28, both the groups demonstrated unification of the osseous tissue. TGF-β staining was positive inside and outside of the osteoblasts, inside of the mature chondrocytes, and was negative inside of the hypertrophic chondrocytes. While there was strong positive staining in the surrounding matrix, the positive staining potency began to weaken. The staining of the SMC group was stronger than that of the DCP group. At day 56, the cellular make up in the osseous tissues in both the groups began to decrease, and the TGF-β staining of the osseous cells and surrounding matrix began to weaken dramatically. The matrix cells on the surface of the endosteum were stained light yellow, in both the groups.

3.2 Quantitative Immunohistochemistry Analysis

TGF-β staining in both the groups peaked at 14 days post-surgery, and decreased at 56 days in reference to staining potency, positive area ratio, and positive index. The differences between the two groups were significant during day 7 to 28 ($P < 0.05$, $P < 0.01$, Table 1), with the SMC group higher than the DCP group in terms of the TGF-β content.

4. Discussion

In recent years, a consensus has emerged that stress takes effect on fracture healing (Ref 10), supported by numerous studies regarding TGF-β expression during fracture healing under stretch stress (Ref 11, 12). In contrast, there are rare studies about the effects of compressive stress on fracture healing. This study measured TGF-β expression at the union of fractures internally fixed by either SMC or DCP using an immunohistochemistry approach. Through histological observation, it was seen that at the protein level, extracellular TGF-β was at the fracture hematoma within 24 h. This process could stimulate proliferation of the local mesenchymal cells, promote collagen synthesis, form granulation tissue, and initiate fracture repair. During the intramembranous ossification stage, the repairing cells themselves began to synthesize TGF-β, represented by positive staining of the mesenchymal cells, osteoblasts, and inside the matrix. Proliferation and differentiation of the bone-producing cells were adjusted through autocrine and paracrine pathways to induce fracture repair. During the chondrification stage, there were some chondrocytes in the callus strongly stained by TGF-β, indicating promotion of synthesis of the cartilage matrix. From the entochondrostosis through the osseous reconstruction, the bone-producing cells positive for TGF-β began to taper, and the TGF-β positive staining potency in the matrix began to weaken, indicating a transformation from cartilage to bone. The fracture healing was invariable under either internal fixation of the SMC or the DCP, and showed positive TGF-β staining in both locations,

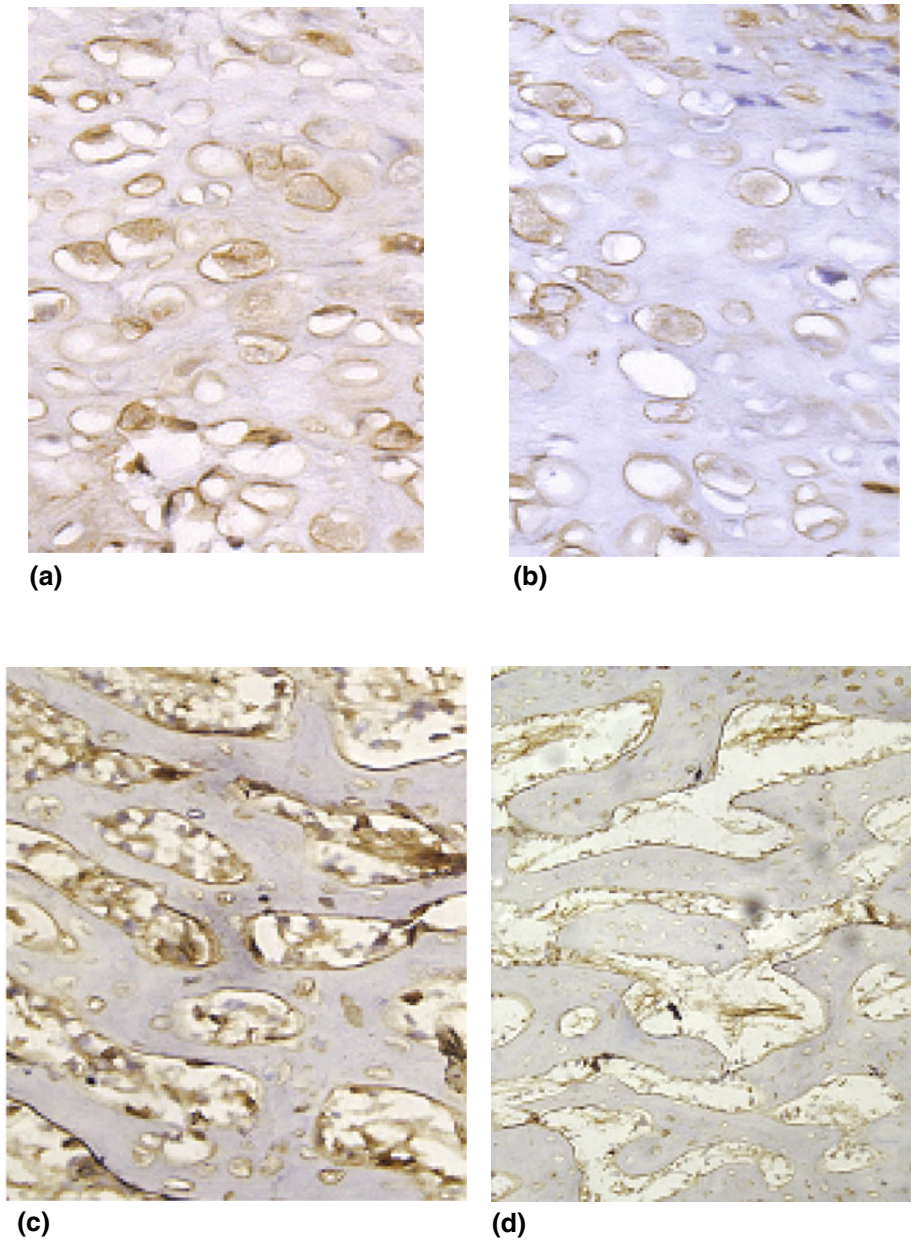

Fig. 1 Expression of TGF-β at fracture sites fixed by SMC (a, c) and DCP (b, d) at 7 days (a, b—magn. 80×) and 14 days (c, d—magn. 40×) after operation (IHC-Envision)

reflecting its participation, consistent with results of previous studies (Ref 6).

Results of this study confirmed that during the chondrification and endochondral ossification stages, TGF-β content in the SMC group was higher than that of the DCP group. This may be explained by the fact that the SMC was made of a nickel-titanium alloy, in which the metallic phase is mutually reversible austenite/martensite and tends to rebound to its original shape in a process driven by body temperature when embedded in vivo, causing a constant pressure across the entire field (Ref 3). The alloy was also super-flexible without a stress shielding effect upon fixation of the fracture (Ref 2). In contrast, the metallic phase of the DCP was austenitic, merely providing once, passive and static pressure upon fixation of the fracture. The force value tended to taper or disappear with the absorption of the fracture line, and furthermore, a stress-shielding effect occurred in the late stage of the fracture fixation. In the early stage of the fracture healing, both the SMC and the DCP are able to provide compressive stress, and the TGF-β in the hematoma of the fractured end is primarily released by platelet degranulation (Ref 9), causing a similar TGF-β expression in both the groups. However, with repair elongating, in the chondrification and early stage of the endochondral ossification, the SMC continues to provide constant and dynamic compression to the fracture. This is due to the prosperous cellular proliferation and differentiation, maintaining its stress stimulation to the bone-producing cells and a high level of synthesis and secretion of the TGF-β. In the late stages of the fracture repair, the tissues in both the groups were similar to those of the normal bones. Although the TGF-β content in both the groups showed a slight descending-ascending-descending trend, the SMC fixation presented a

Table 1 TGF-β positive staining potency, positive area ratio and positive index at fracture sites fixed by SMC and DCP. ($n = 5$, $x + s$)

	Time, t/day						
Index	**1**	**3**	**7**	**14**	**21**	**28**	**56**
Positive staining potency							
SMC	3.77 ± 0.23	4.39 ± 0.30	10.11 ± 1.25*	13.41 ± 1.25**	12.74 ± 1.27**	7.40 ± 0.64**	2.86 ± 0.31
DCP	3.63 ± 0.25	4.31 ± 0.17	8.32 ± 0.72	10.02 ± 0.87*	8.88 ± 0.77	5.25 ± 0.57	2.31 ± 0.29
Positive area ratio							
SMC	0.19 ± 0.02	0.14 ± 0.02	0.13 ± 0.02*	0.17 ± 0.03*	0.13 ± 0.02*	0.09 ± 0.03*	0.04 ± 0.03
DCP	0.20 ± 0.03	0.12 ± 0.02	0.09 ± 0.02	0.13 ± 0.03	0.10 ± 0.02	0.06 ± 0.01	0.03 ± 0.02
Positive index							
SMC	0.74 ± 0.12	0.61 ± 0.09	1.30 ± 0.27*	2.35 ± 0.61**	1.59 ± 0.18**	0.66 ± 0.22*	0.12 ± 0.10
DCP	0.74 ± 0.10	0.51 ± 0.06	0.79 ± 0.21	1.30 ± 0.34	0.85 ± 0.22	0.33 ± 0.10	0.10 ± 0.06

* $P < 0.05$, ** $P < 0.01$ vs. DCP group

healing speed, union quality, and force strength superior to those of the DCP (Ref 3, 13).

5. Summary

In summary, SMC fixation promotes synthesis and secretion of TGF-β in the process of fracture healing and is conducive to endochondral ossification, which may be due to a constant and dynamic compression provided by the SMC.

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