Expression and significance of related genes in the early stage of post-traumatic heterotopic ossification in a rat model of Achilles tenotomy

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ABSTRACT

Objective: This study aims to determine expression profiles of relevant genes in the early stages of post-traumatic heterotopic ossification (HO) in a rat model of Achilles tenotomy.

Methods: A total of 80 male Sprague-Dawley rats were randomly assigned to two groups: the HO group and the control group. Tenotomy was performed in the Achilles tendon of the rats in the HO group, and no intervention was conducted in the control group. On the 3rd, 5th, 8th, and 14th days after the operation, 8 rats were taken from each group at each time point, and the Achilles tendon and surrounding tissue specimens were collected. Gene expressions of TGF-β, BMP, GDF, IL, and MMP families as well as TNF-α, HIF-1α, chordin, gremlin, noggin, and NODAL were analyzed by qRT-PCR. The relevant genes that were highly expressed at different time points were screened, and immunohistochemical staining was then used to verify their expression. At the 10th week, HO formation was explored by radiographic and histological examination in the remaining 8 rats of each group.

Results: Both the radiographic and histological analyses indicated that all the rats developed HO in the HO group (100%), whereas no HO occurred in the control group. Surrounding tissues obtained from the HO group showed significantly higher gene expressions of TGF-β1, BMP-1, IL-1β, HIF-1α, and MMP-2 but lower expressions of BMP4, GDF-6, and TNF-α compared with the control group. In addition, immunohistochemical staining confirmed the higher protein expression levels of relevant genes in the HO group.

Conclusion: TGF-β1, BMP-1, IL-1β, HIF-1α, MMP-2, BMP4, GDF-6, and TNF-α may be associated with the formation of traumatic HO and BMP4, GDF-6, and TNF-α may play a protective role in the early stage of HO. In this study, we investigated the expression levels of the related cytokines in the early stages of traumatic HO in the Achilles tendon tenotomy rat model to better understand the pathogenesis of HO.

Introduction

Acquired heterotopic ossification (HO) is a pathological phenomenon of mature bone tissue formation in soft tissues because of severe trauma, extensive burns, or injuries of the central nervous system (1-3). The common prevention or treatment methods of HO include nonsteroidal anti-inflammatory drugs, radiation therapy or surgical resection (4-7).

Previous studies have demonstrated the promoting role played by transforming growth factor-β and its family members in the formation of HO (8). Sawyer et al. have found that the content of TGF-β1 was 6.8 times higher in HO than in the normal bone in an age-matched study (9). BMP-2, BMP-4, and BMP-7 have also been shown to induce heterotopic bone formation in vivo (10, 11). Despite continued efforts to determine the cellular and molecular events that lead to HO, the exact signaling pathways in the pathogenesis of HO remain elusive. Moreover, many new factors have attracted wide attention, such as inflammation, hypoxia, extracellular matrix remodeling, etc. (2, 12, 13). Evans et al. have found that the high expressions of IL-6, IL-10, and MCP-1 could be used as good indicators for the early diagnosis of HO (12). Studies have also shown post-traumatic HO formed by endochondral ossification in which angiogenesis, bone formation, and extracellular matrix remodeling were essential (13-15). It has been reported that HIF-1α participates in the recruitment of mesenchymal stem cells, regulates the differentiation of mesenchymal stem cells into cartilage, and promotes angiogenesis (2, 14, 16). Furthermore, matrix metalloproteinases (MMPs) can effectively degrade the extracellular matrix and basement membrane and promote angiogenesis (17). Oliveira et al. have suggested that MMPs were also involved in bone remodeling and angiogenesis, which could be considered as a potential biomarker of HO (18).

In this study, we investigated the expression levels of the related cytokines in the early stages of traumatic HO in the Achilles tendon tenotomy rat model to better understand the pathogenesis of HO.

Materials and Methods

Animal experiments

All animal protocols were approved by the Animal Care and Use Committee of East China Normal Uni-
The rats in the control group underwent the same type of skin incision, and no intervention was done on the Achilles tendon, and the wound was sutured back. Perioperative analgesia was mainly acetaminophen syrup for 1-2 days. After the operation, all rats were housed in separate cages with free access to food and water without immobilization.

On the 3rd, 5th, 8th, and 14th days after the operation, 8 rats were taken from each group at each time point and sacrificed by injecting a large dose of chloral hydrate. The Achilles tendon and surrounding soft tissues were obtained by reentering through the original incision. The specimens were divided into 2 parts, of which 1 part was immediately collected and put into a freezing tube, which was then frozen in liquid nitrogen and stored at −80 °C for use for real-time PCR evaluation. The other part was fixed in neutral formalin (10%) for 2 days, followed by decalcification in 15% EDTA for 7 days used for histological and immunohistochemical staining. In the 10th week after the operation, the remaining 8 rats in each group underwent X-ray imaging for the evaluation of the formation of HO, and then the specimens were collected for histological staining.

Real-time polymerase chain reaction

We quantified the gene relative expression of transforming growth factors TGF-β1, TGF-β2, and TGF-β3; bone morphogenetic proteins BMP-1, BMP-2, BMP-4, BMP-5, BMP-7, and BMP-9; growth and differentiation factors GDF-5, GDF-6, GDF-10, and GDF-11; interleukins IL-1β, IL-6, and IL-13; tumor necrosis factors TNF-α; matrix metalloproteinases MMP-2, MMP-9, and MMP-13; hypoxia-inducible factor HIF-1α; chordin (CHRD); gremlin (GREM); noggin (NOG); and NODAL.

The total RNA of the specimens was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and confirmed to the US National Institutes of Health guidelines. A total of 80 male Sprague-Dawley rats were randomly divided into the experimental group (HO group) and the control group. All rats were anesthetized with a peritoneal injection of pentobarbital sodium (3 mg/mL; 1 mL/100 g; Sigma-Aldrich; USA), followed by the preparation of the skin of the posterolateral right leg for surgery. Ethanol (75%) was used to disinfect the surgical site. In the HO group, the Achilles tendon was exposed with a longitudinal incision in the right hind limb. It was then clamped with a pair of forceps 10 times and completely severed at the midpoint without any suture. The incision was closed with a 4-0 silk suture.

All samples were run in triplicate in each experiment. Primers for PCR are presented in Table 1. The PCR cycling conditions were as follows: 95 °C for 5 min; followed by 40 cycles at 95 °C for 15 s, and 60 °C for 60 s. Gene expression levels of mRNA were normalized to GAPDH using the comparative cycle threshold (Ct) method.

**Histological and immunohistochemical staining**

Paraffin-embedded sections were cut into 5 μm sections and then deparaffinized, hydrated, and stained with hematoxylin-eosin (H&E). H&E staining was performed on a number of sections for light microscopy (TS100, Nikon, Japan).

For immunohistochemical staining, the paraffin sections were deparaffinized and hydrated through graded alcohols. The corresponding antibodies were diluted in phosphate-buffered saline (PBS) as follows: BMP-1 (Abcam, Catalogue No. ab205394, Cambridge, UK), 1:400; TGF-β1 (Abcam, Catalogue No. ab64715, Cambridge, UK), 1:400; IL-1β (Abcam, Catalogue No. ab106278, Cambridge, UK), 1:400; HIF-1α (Abcam, Catalogue No. ab160666, Cambridge, UK), 1:400; MMP-2 (Abcam, Catalogue No. ab37150, UK), 1:400. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 30 min. After blocking with goat serum (1:100) for 1 h and incubation with the primary antibody at 4 °C overnight, the sections were washed with PBS. The labeled secondary anti-rabbit IgG (Sigma Aldrich, Catalogue No. A6154, USA) was added and incubated for 1 h at 37 °C. DAB was used for visualization, after which the sections were rinsed with PBS and then counterstained with hematoxylin. Negative sections were incubated in PBS without the primary antibody. All sections were examined under a light microscope (TS100, Nikon, Japan).

**HIGHLIGHTS**

- Rat Achilles tendon tenotomy model is a reliable heterotopic ossification model.
- TGF-β1, BMP-1, IL-1β, and HIF-1α are highly expressed in the formation of traumatic heterotopic ossification.
- BMP4, GDF-6, and TNF-α may play a protective role in the early stage of heterotopic ossification.

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**Table 1. Sequences of oligonucleotide primers used for quantitative real-time qRT-PCR**

| Genes       | Forward Primer (5’-3’)                                                                 | Reverse Primer (5’-3’)                                                                 |
|-------------|---------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| TGF-β1      | CTGCCGTGACCTGTAGTGC                                                                     | GCTTATGCTGGACGATGCCGTCG                                                               |
| TGF-β2      | TCGAGTACTTATGTGC                                                                      | CCGTGACGTGGACGATGCCGTCG                                                               |
| TGF-β3      | CTGCAGTACCTGTAGTGC                                                                    | CGCGGATGCAGTGACGTCG                                                                  |
| BMP-2       | GGCCAGCCGCTCTCTGCTCCTAGT                                                             | TCACTACTATTGGCTGGACGTCG                                                              |
| BMP-4       | TTGGATACCTGGACTAGGCGGCAGAGG                                                          | AACACTGACTAGGCGCTGGACG                                                               |
| BMP-5       | TTACTTCGTGACTAGGGATTGGCTTGG                                                        | TGGAGACTAGGCGCTGGACG                                                                |
| BMP-7       | AGGCAGAGGCGCTTTCTTGTCC                                                               | ATGTCGTGATGGCGCTGGACG                                                               |
| BMP-9       | CAGAAGCTGGAGAAGCGAGTGGT                                                             | CAGGCAGTGGAGCGCTGGACG                                                              |
| GDF-5       | TTGCTGCTGGACATGAGGAGAGG                                                            | ACAAGCTGATAGGACGTCGAGAGG                                                           |
| GDF-6       | TTAACTCCGGCCTGAGGAGAAGA                                                             | CCTGCCTGAGCTTGAGTGGT                                                                |
| GDF-10      | TCTGCTGAGGATGAGGAGGAGG                                                             | GCTGGCTGAGCTTGAGTGGT                                                                |
| GDF-11      | CCTACGGCAAGACGAGCAAGAGG                                                           | GCCACCTTCTCTGCTGGT                                                                |
| IL-1β       | AAAAGCTGCTGCTGCTGCTGCTG                                                          | TTCCTGCCTTCTCTGCTTG                                                                 |
| IL-6        | AGTGCCTCTCTGCTGCTGCTG                                                              | ATGTTCTGCTGCTGCTGGT                                                                |
| IL-15       | CATCCACTGCTGCTGCTGCTGCTG                                                          | GCCCTGCTTGTGCTGCTGGT                                                                |
| TNF-α       | AGACACGCACTCAGAGTGGT                                                               | TGGATCCTGCTGCTGCTGGT                                                                |
| MMP-2       | CACGACGAGAGGATGAGAGGAGG                                                            | GCCACCTGCTGCTGCTGCG                                                                |
| MMP-9       | CGTCCCTTCTCTCTGCTCTCTC                                                  | AAAACCCACTGCTGCTGCTGCG                                                             |
| MMP-13      | CTTGTGCTGCTGCTGCTGCTGCTG                                                        | GCTGCTGCTGCTGCTGCTG                                                               |
| HIF-1α      | CACCTTCTCTCAGAGTGGT                                                               | GCACTGCTGCTGCTGCTGCTG                                                             |
| CHRD        | CACCTGCTGCTGCTGCTGCTGCTG                                                          | GCACTGCTGCTGCTGCTGCTG                                                             |
| GREM        | GCCACCTCTGGGAGAGAGGAGG                                                            | CAGTGCTGCTGCTGCTGCTG                                                              |
| NOG         | GCCACCTCTGGGAGAGAGGAGG                                                            | GCCACCTCTGGGAGAGGAGG                                                              |
| NODAL       | AGAGCTGAGCGCTGCTGCTGCTG                                                          | AGGAGCTGAGCGCTGCTGCTG                                                             |
| GAPDH       | AAGTGCTGCTGAGGAGGAGGAGG                                                            | CAGTGCTGCTGCTGCTGCTG                                                              |
| GAPDH       | AAGTGCTGCTGAGGAGGAGGAGG                                                            | CAGTGCTGCTGCTGCTGCTG                                                              |
Radiological examination

The rats from the control and experimental groups were anesthetized at 10 weeks post operation using the method described above. Lateral right hind limbs were examined by radiography (Kodak, NY, USA) to observe the formation of HO.

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences version 19.0 software (IBM SPSS Corp.; Armonk, NY USA). As data were not normally distributed, all data were presented as median (interquartile range). Statistical differences between the groups were evaluated using the Mann–Whitney U test. The level of significance was set at p<0.05.

Results

Heterotopic ossification at the 10th week

Ten weeks after the operation, the rats in the HO group and control group were subject to an X-ray examination. HO was observed in the Achilles tendon of all 8 rats in the HO group (100%), and there was no evidence of HO in the 8 rats of the control group (Figure 1. a, b). HE staining of the Achilles tendon tissues in rats of the HO group showed visible mature bone, trabecular bone, and cartilage cells, whereas the HE staining of the Achilles tendon in rats of the control group showed normal, regular connective tissue with no indication of bone tissues and cartilage cells, which was consistent with the results of X-ray diagnosis of HO (Figure 1. c, d).

Gene expressions after Achilles tenotomy

In the HO group, TGF-β1, TGF-β2, and TGF-β3 were highly ex-
pressed, and only the expressions of TGF-β1 at each time point were statistically significant; the expressions of TGF-β2 and TGF-β3 on the 5th and 8th days after the operation were statistically significant. Compared with the control group, BMP-1 was the only factor that was highly expressed in the BMP family at each time point, and the expressions were statistically significant. In the HO group, the expression of BMP-2 was significantly lower on the 3rd day, whereas the expressions of both groups showed no significant difference at other time points. The expressions of BMP-4 in the HO group at each time point were down-regulated, and the differences were statistically significant. BMP-5 had a lower expression, and only the expressions on the 3rd and 8th days after operation were statistically significant. BMP-7 and BMP-9 were highly expressed, and only the expressions on the 3rd and 5th days were statistically significant. CHRD, GREM, and NOG were the antagonists of BMP, and they had lower expressions in the HO group, which were statistically significant on the 3rd day. BMP-10 was the only factor that showed low expression in the GDF family, and the expressions were statistically significant at each time point. GDF-5 and GDF-11 were highly expressed, and only the expressions on the 3rd and 5th days were statistically significant.

Histological staining
At each time point, HE staining showed that the tendon tissues in the control group were normal, regular connective tissues. By contrast, in the HO group, a large number of inflammatory cells entered into the Achilles tendon tissues from the blood vessels. On the 3rd, 5th, and 8th day after the operation, a large amount of inflammatory cell infiltration was observed, including monocytes, macrophages, and fibroblasts. On the 14th day after the operation, a larger amount of inflammatory cell infiltration was observed, accompanied by fibrous connective tissue formation, indicative of a reparative response in the tissue (Figure 5).

Protein expression levels after Achilles tenotomy
Immunohistochemistry was used to confirm the statistically significant and substantially up-regulated cytokines in the Achilles tendon tissue. In the control group, TGF-β1, BMP-1, IL-1β, HIF-1α, and MMP-2 showed low expressions, or the expressions were not detected. In contrast, in the HO group, TGF-β1, BMP-1, IL-1β, HIF-1α, and MMP-2 were highly expressed at each time point (Figures 5 and 6).

Discussion
This study aimed to investigate the expressions of the related factors in the early stages of traumatic HO in the Achilles tendon tenotomy.
Figure 4. Relative gene expression levels of the factors related to HO detected by real-time PCR on the 3rd, 5th, 8th, and 14th days after operation. IL-1β, IL-6, IL-15, TNF-α, MMP-2, MMP-9, MMP-13, and HIF-1α. *p<0.05.

Figure 5. Observation of the HE staining, BMP-1 immunohistochemical staining, and TGF-β1 immunohistochemical staining of Achilles tendon tissues in the two groups on the 3rd, 5th, 8th, and 14th days after operation. The black arrow indicated the positive staining.
The Achilles tendon tenotomy model could induce HO in a relatively short period with similar morphology and imaging to the pathological changes observed in HO caused by trauma in clinical settings. Furthermore, the animal model had the advantage of easy operability, high success rate, and good reproducibility. Therefore this process has been widely used in many studies of HO (19, 20).

In this study, the incidence rate of HO was 100% in the HO group at 10 weeks after the operation.

The results of the study showed that TGF-β₁ was highly expressed at each time point in the early stages of HO. A study has shown that TGF-β₁ can induce endothelial cells to differentiate into mesenchymal stem cells before the formation of HO, enhance the differentiation potential of neighboring mesenchymal stem cells, and initiate its differentiation into osteoblasts (21). Some scholars have also pointed out that TGF-β₁ can activate Smad2 and Smad3 and increase the expression of SOX-9, resulting in the differentiation of stem cells to the cartilage stage. TGF-β₁ is thought to play multiple roles in the development and progression of HO because of its ability to promote muscle fibrosis and osteogenic induction (22).

BMP-1 was significantly up-regulated at each time point of traumatic HO and could play an important role in the degradation of collagen precursor, inactivation of BMP antagonists, and activation of TGF-β₁. Maeda et al. found that in the process of osteoblast differentiation, there was positive feedback between TGF-βs and BMPs (23). BMP-4 was significantly down-regulated in the early stages of traumatic HO, indicating that it was involved in traumatic HO; however, the specific mechanism of action remains to be further investigated.

GDFs are a member of the TGF-β superfamily. In the early stage of traumatic HO, GDF-8 was the only factor that was down-regulated among the GDFs, and the expressions were statistically significant. A study has pointed out that GDF-8 can inhibit the proliferation of myoblasts and play a negative regulatory role in the development and differentiation of muscle (24). In this study, although the role of GDF-8 in HO was uncertain, it is believed that GDF-8 may play a protective role in the early stage of traumatic HO.

The results of the study showed that IL-1β was significantly up-regulated in the early stage of traumatic HO. Moreover, HE staining showed a large number of inflammatory cell infiltrations (neutrophil granulocytes and macrophages) in the HO group, indicating that inflammatory reaction may be an important part of the development and progression of HO. Some scholars have pointed out that IL-1β can activate nuclear factor-κB (NF-κB) and a variety of downstream genes, such as the overexpression of MMPs (25). MMPs play a crucial role in the process of angiogenesis, extracellular matrix degradation, remodeling, and calcification (26). Evans et al. further found that IL-6 was significantly higher in the serum of patients with severe trauma, which can be used as an indicator of the occurrence and severity of HO (12). However, our results showed that the expression of IL-6 was not significantly increased in the early stage of traumatic HO. In addition, in the early stage of traumatic HO, the expression of TNF-α was significantly down-regulated, which was also contrary to the result of Jackson et al. [22]. However, Luo found that TNF-α was significantly down-regulated in patients with HO, but it was significantly up-regulated in the specimens of patients without HO. When the expression of TNF-α increased, it inhibited the expression of BMP-2, and it is believed that TNF-α, as an inflammatory factor, inhibited osteoblasts and stimulated osteoclasts, and played a protective role in the development of HO (8).
In recent years, it has emerged that HIF-1α plays an important role in the pathogenesis of HO, and its function may be involved in the accumulation of mesenchymal stem cells, angiogenesis, and bone formation (27-29). In our study, the gross observation and histological staining showed that on the 3rd and 5th days after the operation, the atrophy of the Achilles tendon terminal and the peripheral tissue ischemic necrosis occurred in the rats of the HO group, indicating that the traumatic Achilles tendon and the surrounding tissues in the early stage were in an anoxic or extreme hypoxic microenvironment, thus justifying the high content of HIF-1α in the local Achilles tendon tissues of the HO group by qRT-PCR, which was significantly higher than that of the control group at each time point. Immunohistochemical staining also showed that the expression of HIF-1α in the local Achilles tendon tissues of the HO group increased, confirming the view that HIF-1α plays an important role in the early stage of traumatic HO when the Achilles tendon tissues are in a hypoxic microenvironment. Lin et al. have believed that the high expression of HIF-1α can directly promote the expression of SOX-9 and induce the differentiation of HO precursor cells into cartilage cells in a hypoxic microenvironment; moreover, it regulated the expression of Runx-2 by BMP/SMAD signaling pathway and promoted osteoblastic differentiation (20). They suggested that VEGF can amplify the BMP/SMAD signaling pathway and eventually lead to the formation of HO. Agarwal et al. have expounded that the high expression of HIF-1α may regulate the expression of SOX-9 via the BMP/SMAD signaling pathway and that the expression of SOX-9 had a synchronous relationship with HIF-1α (30).

MMP is mainly secreted by inflammatory cells, vascular endothelial cells, and glial cells, and a variety of stimulating factors (TGFβ, IL-1, TNF-α, etc.) can activate its expression (31). QRT-PCR analysis showed that in the early stage of traumatic HO, the expression of MMP-2 was significantly up-regulated in local tissues, confirmed by the immunohistochemical staining. This demonstrates that MMP-2 may be involved in the development of HO, an early marker of the pathological development of HO.

The large amounts of inflammatory cell infiltrations and high expressions of HIF-1α and IL-1β in the early stage indicated that the traumatic HO in the early stage was mainly in a hypoxic and inflammatory microenvironment. HO microenvironment is a complex integrated system, which is mainly composed of the following aspects:

Physicochemical properties namely hypoxia, ischemia, and an acidic microenvironment.

- Presence of HO-related stromal cells including fiber cells, inflammatory cells, vascular endothelial cells, etc.
- Additional presence of a variety of cytokines, chemotactic factors, neuropeptides, etc.
- Activation of extracellular matrix proteins and their receptors including collagen, matrix metalloproteinases, etc.

Agarwal et al. used animal in vivo imaging technique to verify that the rat Achilles tendon was in a hypoxic state in an Achilles tendon tenotomy/burn model (30). Most researchers have also confirmed the inflammatory state in the HO microenvironment (12, 32). This study suggested that the early microenvironment of traumatic HO may be characterized by hypoxia, inflammation, and high expression of specific factors.

This study has some limitations. First, the results of gene expression and histological analysis were obtained after the collection of all specimens at 10 weeks after the establishment of the HO model; therefore, the effects of the time interval between the experimental operation and specimen collection on the outcomes were not considered in the study. Second, we just performed an IHC examination about the up-regulated molecules but did not include all the molecules that showed expression difference.

In conclusion, it is believed that TGF-β1, BMP-1, IL-1β, HIF-1α, MMP-2, BMP-4, GDF-6, and TNF-α were associated with the formation of traumatic HO, and BMP-4, GDF-6, and TNF-α might play a protective role in the early stage of HO. However, the roles of these factors in the early stage of traumatic HO and their molecular mechanisms need further investigation in animal models and in vivo human studies.

Ethics Committee Approval: Ethics committee approval was received for this study from the East China Normal University (protocol number: AR2016/11037).

Informed Consent: N/A.

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