Characterization of cytokines expression and relative signaling molecules after condylar fracture

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Received September 14, 2017; Accepted July 1, 2018; Epub October 15, 2018; Published October 30, 2018

Abstract: Aim: To characterize the expression profiles of cytokines, including FGF1 (fibroblast growth factor 1), IGF-1 (insulin-like growth factor 1), IL-1 (interleukin 1), IL-6 (interleukin 6), TGF-beta (transforming growth factor 1 beta), BMP (Bone morphogenetic proteins) as well as PI3K (phosphoinositide 3-kinase) and AKT (protein kinase B) after condylar fractures. Methods: 15 healthy goats aged 6-8 months, weighed 10-12 kg were used for establishing the condylar fracture model through hitting the temporomandibular joint region in one side. Condylar facture was validated by immediate CT scanning. Cartilage tissues in temporomandibular joint region from both fractured and un-hit control side were dissected for measuring the cytokine expression by immunohistochemical staining, western blot and RT-PCR. Results: The expression of BMP was 101.51 ± 2.156 in the experimental side and 101.23 ± 1.895 in the control side without significant difference (P = 2.64). Meanwhile, there was also no significant difference in the expression of FGF1 (P = 3.36) between the control side (91.03 ± 2.365) and the experimental side (91.19 ± 3.128). However, compared with the control side (97.26 ± 1.017), the expression of IGF1 in the experimental side (146.04 ± 1.235) was significantly higher (P = 0.001). In addition, the expression of IL-1beta (146.04 ± 1.235 vs 98.77 ± 1.826, P = 0.001) and IL-6 (130.08 ± 1.032 vs 83.05 ± 1.223, P = 0.001) was also significantly higher in experimental side than those in control side. Interestingly, no significant difference of TGF-beta expression was observed between experimental and control side (P = 4.43). However, the mRNA and protein expression levels of PI3K and AKT were significantly higher than those in healthy controls (P = 0.001). Conclusion: Enhanced expressions of IGF-1, IL1-beta, IL6, PI3K and AKT were observed in condylar fracture progression, suggesting targeting them might be a novel approach to promote the repair after condylar.

Keywords: Condylar fractures, cytokine, IL-1beta, IL-6, IGF-1, PI3K/AKT, IGF-1 signaling

Introduction

Condylar fracture is a common type of bone fracture in mandible, with incidence of one-third among total mandible fracture [1-4]. Thus it’s of great importance to explore the biological events during recovering of condylar fracture, which might provide guidance on possible interventions to improve patients’ outcome.

Bone and immune system are tightly related and inflammatory disorders are associated with bone loss [5-8]. Tissue damage and blood vessel rupture resulting from fracture can promote immune response [9, 10]. During fracture repair process, inflammatory responses are also initiated and contribute to the repair process with involvement of multiple cellular events within multiple cell types [11, 12]. Balanced inflammation at the fracture site can restrict tissue damage and initiate tissue repair through producing pro-angiogenic mediators and attracting mesenchymal progenitors cells, and is crucial for fracture healing [12, 13]. In contrast, fracture healing is disturbed when the inflammatory response is increased or prolonged [14]. Thus inflammatory responses are important for the recovery after bone fracture.

In the current study, we aimed to explore the possible involvement of immune response in condylar fracture goat model through measuring the expression profiles of cytokines, which play important roles in the regulation of immune response, including IL-1beta [15], IL-6 [16], IGF-1 [17], TGF-beta [18], and the related signaling molecules. Our study will provide clues of the involvement of cytokines in condylar fracture, which may provide theoretic basis for developing new treatment approach to promote
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recovery from condylar fracture through targeting immune response.

Methods

Sample preparation

15 healthy goats aged 6-8 months and weighed 10-12 kg were purchased and housed in the Animal Experiment Centre of the First Affiliated Hospital of Xinjiang Medical University. To produce condylar fractures, temporomandibular joint region of one side was hit to produce fracture which was further validated by immediate CT scanning (Figure 1). Cartilage tissues were resected from both the fractured and un-hit control under general anesthesia induced with Ketamine via intramuscular injection. The operation followed protocols of the Yang Chi’s Experimental Approach from the Ninth Affiliated Hospital of Shanghai Jiaotong University in which the joint zone of the fracture was exposed by a side incision during which the cartilage of the condylar surface was exposed by separating. Same approach was applied for resecting for control preparation. All procedures were approved by the Ethic committee.

HE staining

1 month after fracture, the joint cartilage and crystals were collected and fixed with 10% formalin overnight for paraffin embedded section. Section was then deparaffined in Xylene for 2 X10 min. After infiltration in absolute alcohol for 5 min, 90% ethanol for 2 min and 70% ethanol for 2 min, sections were placed in distilled water for 2 min, stained with eosin for 2 min and then washed in tap water to remove remaining staining solution. Sections were then stained with Hematoxylin for 10 min and washed with tap water to remove remaining staining solution. After that, sections were further immersed in gradient ethanol of 70%, 90% and 100% for 2 min, 2 min and 5 min respectively.

Immunohistochemical staining

Deparaffined sections were burned with mid and high fire for 3 min respectively for antigen repair. After blocked in blocking solution for 2 hours at 37°C, sections were incubated with primary antibodies against BMP, FGF1, IGF1, IL-1beta, IL-6 or TGF-beta (Santa Cruz Biotechnology, Dallas, Texas, USA) overnight at 4°C. On the following day, sections were washed with PBS for 3X 5 min and then incubated with HRP-conjugated second antibody (1:1000) (Santa Cruz Biotechnology) for 2 hours at 37°C. DAB was applied for antigen visualization after incubation for 30 min-1 hour and remaining DAB solution (Thermo Fisher Scientific, Waltham, MA, USA) was washed out with tap water when signals were visible under a microscope. Sections were then further re-stained with Hematoxylin, dehydrated for transparency and examined with neutral resin sheet.

Western blot test

Protein level of PI3K and AKT was detected by Western blot followed standard protocol. In
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brief, total protein was extracted using RIPA lysis buffer (Thermo Fisher Scientific) from tissues and quantified by BCA. 40 μg protein was separated by 10% SDS-PAGE and transferred to PVDF membrane (Gibco, Grand Island, NY, USA). After that, the membrane was blocked and incubated with primary antibodies (all from Santa Cruz Biotechnology) at 4°C overnight (PI3K, AKT and β-actin at 1:500, 1:500 and 1:800, respectively). Then the membrane was incubated with HRP-conjugated secondary antibody (1:5000) (Santa Cruz Biotechnology) for 60 min after washed by PBST for three times. At last, the protein expression was detected by ECL chemiluminiscence (Thermo Fisher Scientific).

RT-PCR experiment

The reverse transcription reagent kit and the RT-PCR reagent kit were purchased from TaKaRa and all procedures were performed according to the manufacture's manual. Primers for RT-PCR were as follows: AKT forward: 5’-G-GCCCATATGATCACCCTAC-3’ & AKT reversed: 5’-CTATCGTCACGCGAAGTCCA-3’; PI3K forward: 5’-ACATGGAACCCTCAGTTACACAA-3’ & PI3K reversed: 5’-ACTGGAAACACAGTCCA-TGCACATA-3’.

Statistical analysis

Statistical analysis was performed using SPSS software (version 20.0). All data was presented as mean ± standard deviation (SD) and analyzed by ANOVA. Independent t-test was used to compare the significance among the two groups. P < 0.05 indicates a statistical significance.

Result

Disrupted cellular arrangement after fracturing under HE staining

HE staining showed that cartilage and cartilage cell displayed light pink and blue, respectively. In control samples, cartilage cells lined orderly into upper, middle, columnar and cartilage layers (Figure 2A), whereas, in fractured samples, the arrangement of cell appeared to be disrupted (Figure 2B).

Expression of cytokines

The expression of BMP in experimental side was 101.51 ± 2.156, without significant differ-
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The protein and mRNA expression of PI3K and AKT

To uncover the possible mechanisms underlying the elevated expression of cytokines, we measured the expression of PI3K and AKT to assess the status of PI3K/AKT signaling. We found that the mRNA (Figure 5A and 5C) and protein (Figure 5B and 5D) level of PI3K and AKT was increased significantly in fractured compared with control samples (P < 0.05), suggesting elevated cytokines expression may result from activated PI3K/AKT signaling.

Discussion

In this study, we successfully established a condylar fracture model in goat (Figures 1 and 2). To investigate the possible contribution of inflammatory response in disease progression, we compared the expression of classic cytokines in fractured samples with those in control samples. We found among BMP, FGF1, TGF-beta, IL-1beta, IL-6 and IGF-1, the expression of IL-beta, IL-6 and IGF-1 was increased significantly in fractured samples (Figures 3 and 4), indicating possible involvement of inflammatory responses in disease progression. Moreover, we found that the mRNA and protein level of PI3K and AKT was increased significantly in fractured samples, suggesting activated PI3K/AKT signaling may contribute to the increased cytokines’ expression. Thus we revealed possible involvement of cytokines in condylar fracture in which elevated PI3K and AKT may play certain roles.

IL-1 plays multiple and diverse roles in inflammatory responses that can regulate the synthesis of IL-2, IL-6 and IL-8, which can increase IL-6 expression, thus forming a positive feedback to enhance the immune reaction and inflamma-

Figure 4. Expression of IGF1 (A & B), IL1-beta (C & D) and IL6 (E & F) in control (left panels) and fractured samples (right panels) (x 40). Deparaffined sections were burned for antigen repair and blocked followed by incubation with primary antibodies and subsequent secondary antibody for immunohistochemical staining.

ence to that in the control side (101.23 ± 1.895) (P > 0.05) (Figure 3). Meanwhile, no significant difference of the expression of FGF1 (91.03 ± 2.365 for control vs 91.19 ± 3.128 for experimental) was found between the two groups (P > 0.05) (Figure 3). However, a significantly higher expression of IGF1 (146.04 ± 1.235 vs 97.26 ± 1.017), IL1beta (146.04 ± 1.235 vs 98.77 ± 1.826) and IL6 (130.08 ± 1.032 vs 83.05 ± 1.223) was observed in experimental side than those in control side (P < 0.05) (Figure 4). However, no significant difference of TGF-beta expression was found between the experimental and control group (P > 0.05) (Figure 3).
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Hong-qiang Liu found that IL-1 could induce the degradation of matrix surrounding cartilage cells and apoptosis of cartilage cells in wet arthritis [20]. Consistent with this, our study showed increased IL-1beta level in fractured side, further confirming the role of IL-1beta in the progression of condylar fracture. IL-6 is an important pro-inflammatory cytokine with many biological activities. It can inhibit the differentiation of bone marrow mesenchymal stem cells into chondrocytes [21]. In addition, IL-6 plays an important role in the cartilage repair [22]. In our study, we found significantly increased IL-6 level in fractured side, revealing the contribution of IL-6 to the cartilage repair after fracturing. IGF-1 is also an important cytokine in promoting cell proliferation and inhibiting apoptosis in several malignant tumors [23, 24]. However, its role in post fracture recovery process remains poorly understood. In this study, we found IGF-1 level was increased after fracturing, indicating it might also participate in the progression of condylar fracture. PI3K/AKT signaling is considered to be an important pathway in regulating the proliferation of malignant tumors, cell metabolism, cycle control as well as the formation of blood vessels [20, 25]. In the present study, we demonstrated that the protein and mRNA expression of PI3K and AKT were increased after fracturing, suggesting PI3K/AKT signaling might be involved in the development of condylar fracture. The increased PI3K/AKT expression might be due to the higher level of IGF-1 as IGF-1 has been reported to be capable to activate PI3K/AKT signaling [26-28]. In addition, increased PI3K/AKT expression might also be associated with the elevated expression of IL-1beta and IL-6, as a closely relationship has been observed among them [29-33].

In conclusion, enhanced expressions of IGF-1, IL1-beta, IL6, PI3K and AKT were observed in condylar fracture progression, suggesting targeting them might be a novel approach to promote the repair after condylar.

Acknowledgements

This study was funded by The Natural fund of the Xinjiang uygur autonomous region, NO. 2016D01C249.

Disclosure of conflict of interest

None.

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