A pilot trial on the molecular pathophysiology of traumatic temporomandibular joint bony ankylosis in a sheep model. Part I: Expression of Wnt signaling

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Abstract

Objective: To preliminarily investigate the temporal patterns of the endogenous mRNA expression for members of the Wnt signaling and a series of genes regulating bone formation during the development of traumatic temporomandibular joint (TMJ) bony ankylosis in a sheep model.

Methods: Six sheep were used for the induction of bony ankylosis of TMJ. We performed a condylar fracture, excision of the lateral 2/3 disc and serious injury to the glenoid fossa to induce bony ankylosis on the right TMJ. An isolated condylar fracture was performed on the left side. Two sheep were sacrificed at 1 month, 3 months, and 6 months after surgery, respectively. The specimens from the ankylosed joint and the condylar fracture were harvested for RNA extraction respectively. In this report (Part I), only the bony ankylosed samples were used for analysis of gene expressions. The specimens 1 month postoperatively were taken as the control, and the changes of expression of target genes over time were examined by real-time PCR.

Results: mRNA expression of Wnt1, Wnt2b, Wnt3a, β-catenin, Sfrp1, Lrp6, Lef1, CyclinD1, and Runx2 was up-regulated at 3 and 6 months compared with 1 month. The expression of Wnt5a, Sox9, and Osterix was up-regulated with a peak at 3 months, and then fell back to the basal levels at 6 months. The expression of Ocn began to up-regulate until 6 month postoperatively.

Conclusion: Our findings suggested that Wnt signaling was involved in the formation of traumatic TMJ bony ankylosis and thus may be a potential therapeutic target for the treatment of the disease in the future.

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the healing cascade of bone (Tsuji et al., 2006; Alam et al., 2009; Rosen, 2009), has been shown at the site of human delayed union and nonunion (Kloen et al., 2002), although the imbalance between the BMPs and BMP inhibitors exists (Fajardo et al., 2009; Kwong et al., 2009).

For traumatic TMJ bony ankylosis, the biological events and the molecular mechanisms are extremely complex and far from being comprehensively understood. In a sheep model of TMJ bony ankylosis which was established by mimicking the traumatic environment of condylar sagittal fracture, i.e. a type M diacapitular fracture according to Neff et al. (1999) viz. a condylar head fracture without preservation of vertical height according to Loukota et al. (2010), the main type of bone formation through endochondral ossification and a prolonged chondral phase compared to condylar fracture healing were shown by histological analysis, which indicated a similarity between bony ankylosis and delayed bone healing (Yan et al., 2012b). The areas of endochondral bone formation were also recognized in surgical samples of humans (Kim et al., 2009; Pilmane and Skagers, 2011). These results suggest that the signaling molecules involved in fracture healing and bone regeneration may also occur in the traumatic TMJ bony ankylosis.

Wnts are secreted, lipid-modified glycoproteins, and at least 19 Wnt ligands have so far been defined in the human and the mouse (Nusse, 2005). According to whether β-catenin involved in or not, the pathways activated by the Wnts can be divided into canonical and non-canonical Wnt signaling (Westendorf et al., 2004). In the canonical Wnt signaling, β-catenin accumulates in the cytoplasm after the binding of Wnts and the receptor complex (consisting of LRP5/6 and frizzled receptors) on the cell membrane (Silkstone et al., 2008; Secretro et al., 2009), β-catenin then translocates into the nucleus and binds to the transcription factors TCF/LEF, stimulating the transcription of target genes (Silkstone et al., 2008; Secretro et al., 2009). The canonical Wnt signaling controls the differentiation of mesenchymal progenitors into chondrocyte or osteoblast lineages during the embryonic development (Day et al., 2005; Rodda and McMahon, 2006), and regulates the bone mass in postnatal and adult life (Glass et al., 2005). Recent studies reveal that the canonical and non-canonical Wnt signaling play important roles in the process of fracture healing and bone regeneration both in the appendicular skeleton and craniofacial skeleton (Hadjiargyrou et al., 2002; Zhong et al., 2006; Chen et al., 2007; Kakar et al., 2007; Kim et al., 2007; Leucht et al., 2008). In addition, for ankylosing spondylitis, the best-known form of spondyloarthritis, the molecular mechanism underlying ankylosis is hypothesized to recapitulate the process of endochondral bone formation (Lories et al., 2005, 2008; Braem and Lories, 2012; Carter et al., 2012) and the Wnt signaling has been shown to contribute to the progression of the condition (Diarra et al., 2007; Appel et al., 2009; Daoussis et al., 2010; Uderhardt et al., 2010; Heiland et al., 2012; Zhao et al., 2012). Therefore we hypothesize that the Wnt signaling may also involve in the development of traumatic TMJ bony ankylosis.

According to the histological features of the animal model, the development of bony ankylosis can be classified into three phases (Yan et al., 2012b): (1) fibrous-chondral phase demonstrating fibrous tissue and chondrocytes occupied the joint gap at 1 month after surgery; (2) chondral-calcified cartilage phase manifesting abundant chondrocytes, cartilage matrix, and neoformative endochondral ossification in the joint space at 3 months postoperatively; and (3) bone-cartilage phase showing compacted bone bridge in the lateral joint gap and cartilage in the medial joint gap at 6 months after surgery.

The aim of the study was to explore the temporal patterns of the endogenous mRNA expression in the above-mentioned three phases of bony ankylosis for members of the Wnt signaling and a series of genes regulating cartilage formation, bone formation and endochondral ossification.

2. Materials and methods

2.1. Animal model building and tissue harvesting

This study was approved by Animal Welfare Branch of Biomedical Ethics Committee of Peking University. Male sheep, 3-month-old sheep with the average preoperative weight of 20.3 kg (±1.9 kg) were used in this study. Surgery was performed as described previously (Yan et al., 2012b). On the left TMJ simulating a fracture, only an artificial condylar sagittal fracture was made and the disc was preserved. On the right joint simulating bony ankylosis, a sagittal condylar fracture, severe damage to the glenoid fossa and removal of the lateral 2/3 disc were performed to induce bony ankylosis. Time points of tissue harvesting were determined according to the histological stages of the development of bony ankylosis in the sheep model as mentioned above. Two animals were sacrificed at 1, 3, and 6 months after surgery, respectively.

Tissue harvesting was performed under general anesthesia in all cases. For the bony ankylosis-induced side, the TMJ complexes were carefully removed en bloc with a fissure bur and a wide osteotome and then promptly dissected free of soft tissue around the joint. The ankylosed joints were split open carefully by a narrow osteotome, and the newly generated tissues in the joint space, namely bony ankylosed callus, were harvested (Fig. 1A). For the fracture side, the condyle was resected using a fissure bur, and the reparative tissues between the sagittal fracture fragments, namely fracture callus, were harvested (Fig. 1B). Both the bony ankylosed callus and fracture callus were frozen in liquid nitrogen and stored at −80 °C for RNA extraction. In this report (Part I), only the results derived from the bony ankylosed callus, namely the changes of gene expression
over time during the formation of bony ankylosis, were described. The animals were killed with a lethal dose of pentobarbitone sodium by intravenous injection.

2.2. Total RNA extraction and the synthesis of cDNA

The bony ankylosed callus or fracture callus was respectively crushed using a mortar and pestle in liquid nitrogen. The total RNA was extracted from the lysate by the Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. The concentration of each RNA sample was determined spectrophotometrically and the integrity of all RNA samples was monitored on denaturing agarose gel electrophoresis. Reverse transcription were performed with a cDNA synthesis kit (Promega, USA) in a 20 µl reaction system containing 4 µg total RNA.

2.3. Primers design and the identity of products

Primers for each target gene were designed using the software of Primer Premier Version 5.0. For ovine genes that the sequences can be acquired from GenBank, the coding sequences of the genes were taken as templates for primer design. For those ovine genes that their sequences can not be acquired from GenBank on account of a paucity of genomic resources, the mutual sequences of the coding sequences of the corresponding human and bovine genes were taken as templates for primer design based on the fact that only less than 3% of protein-coding nucleotide positions are different between cattle and sheep (Hayashi and Spencer, 2006; Kijas et al., 2006). The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Before the quantitative real-time PCR, the reverse transcription–PCR (RT–PCR) was conducted to verify the reliability of primers using the total RNA isolated from the bone or muscle of sheep. The PCR products were visualized on a 1.5% agarose gel stained with GoldenView™ using Gel Imaging System. When the amplified PCR products manifested as a single band, suitable abundance of expression and the predicted size by electrophoresis, they were sent to BGI Sequencing (Beijing) and sequenced in both directions. The bidirectional sequencing results were spliced and formed the full-length nucleotide sequences of the products. The sequences of the products and the template were then compared by BLAST on Pubmed. The 2 sequences were identified as the same gene when their homology was more than 95%. The bands of partial products are shown in Fig. 2. The primers of target genes confirmed by sequencing and BLAST are shown in Table 1.

2.4. Real-Time PCR

Real-Time PCR was carried out on an ABI Prism 7500 (Applied Bioscience, USA). The reaction system and PCR cycle parameters are the same as previously described (Wu et al., 2010). The housekeeping gene GAPDH was used for normalization of gene expression. Relative gene expression was calculated using the 2^{-ΔΔCt} method, where threshold cycle (Ct) values from duplicate runs were averaged and calibrated in relation to GAPDH Ct values.

2.5. Statistical analyses

Levels of gene expression (fold changes) in bony ankylosed callus at 3 and 6 months were presented in relation to expression levels in that at 1 month. For statistical analyses of data, means were calculated for each time point. Statistical comparisons between the different time points were performed using one-Way ANOVA and Dunnett’s T3 Test (SPSS 17.0). The level of statistical
The expression of Wnt1, Wnt2b, Wnt3a, and Sfrp1 was inclined to increase at 3 months in comparison with that at 1 month, with a maximum 2.0-fold change. The expression of Wnt5a fell back to the basal level at 6 months. For the above 5 genes, the statistical differences were found for Wnt1 (P = 0.046) and for Wnt3a (P = 0.039) both at 6 months (Fig. 3).

3.2. Wnt1, Wnt2b, Wnt3a, Wnt5a, and Sfrp1

The expression of Wnt1, Wnt2b, Wnt3a and Sfrp1 was inclined to increase with the progress of bony ankylosis. At 6 months, the maximum changes for Wnt1, Wnt2b, Wnt3a and Sfrp1 were 6.1-, 2.7-, 8.5-, 6.2- and 2.9-fold, respectively. The Wnt5a expression tended to increase at 3 months in comparison with that at 1 month, with a maximum 2.0-fold change. The expression of Wnt5a fell back to the basal level at 6 months. For the above 5 genes, the statistical differences were found for Wnt1 (P = 0.046) and for Wnt3a (P = 0.039) both at 6 months (Fig. 3).

3.3. Lrp6, β-catenin, Lef1, and CyclinD1

The Lrp6 expression was inclined to up-regulate at 3 months, and then maintain the high level at 6 months. The expression of β-catenin, Lef1, and CyclinD1 tended to increased continuously during the formation of bony ankylosis. At 6 months, the maximum changes for Lrp6, β-catenin, Lef1, and CyclinD1 were 3.4-, 8.5-, 6.2-, and 2.7-fold, respectively. For the above 4 genes, the statistical differences were found for β-catenin (P = 0.046) at 3 months and for Lrp6 (P = 0.049) at 6 months (Fig. 4).

3.4. Alp, Ocn, Col1a1, and Col10a1

The expression of Alp and Col10a1 tended to increase at 3 months in comparison with that at 1 month, and then decreased
apparently but still higher than the basal level. At 3 months, the maximum changes were 4.5 fold for Alp and 28.3 fold for Col10a1. There was no apparent change in the expression of Col1a1 over the ankylosis course. The expression of Ocn began to up-regulate until 6 month postoperatively, with a maximum 20-fold change at 6 months. For the above 4 genes, the statistical differences were found for Alp \((P = 0.028)\) at 3 months and for Ocn \((P = 0.022)\) at 6 months (Fig. 5).

3.5. Runx2, Sox9, and Osterix

The Runx2 expression was inclined to up-regulate continuously at 3 and 6 months, with a maximum 4.5-fold change at 6 months. The expression of Sox9 and Osterix tended to increase at 3 months in comparison to that at 1 month, and then decreased back to the basal level. At 3 months, the maximum changes were 8.4 fold for Sox9 and 2.2 fold for Osterix. The statistical differences were found for Runx2 \((P = 0.045)\), Sox9 \((P = 0.016)\) and Osterix \((P = 0.041)\) at 3 months (Fig. 6).

4. Discussion

With a low incidence of ankylosis after TMJ trauma (Laskin, 1978; Hong, 1990), it is difficult to obtain the human specimens and surgical samples are usually obtained from patients with long-standing or end-stage disease. Therefore animal models are invaluable aids in gaining insights into the molecular and cellular mechanisms of the condition. Partly due to lack of a suitable animal model which can well mimic the microenvironment of TMJ trauma, the molecular pathophysiology that leads to bony fusion of the injured articular surfaces rather than condylar fracture healing with remodeling has not been studied.

To objectively evaluate the existing animal models of TMJ ankylosis, 2 criteria should be taken into account. One is the outcome of the model, namely, does bony ankylosis really occur by the induction method? According to the criterion, only Yan et al. (2012b) and Cheung et al. (2007) succeed, and all of the others have been shown to be fibrous ankylosis (Laskin, 1978; Miyamoto et al., 1999; Oztan et al., 2004; Porto et al., 2008; Li et al., 2009b; Rodrigues et al., 2011), which has been postulated to be a different pathological process compared to bony ankylosis (Yan et al., 2012b).

The other criterion is the conformity of occurrence conditions between the model and human beings. The issue is complicated and controversial. On one hand, although the risk factors predisposing to traumatic TMJ ankylosis are indicated by clinical observations...
other synovial joints that develop by the cleavage or segmentation mesenchymal condensations, which is different signification in the development of traumatic TMJ bony ankylosis, provided preliminary evidences for the potential role of Wnt signaling (Miyamoto et al., 2000; Cheung et al., 2007), highlight its usefulness and reproducibility of true bony ankylosis in our model without bone graft in the joint space in contrast to other models (Miyamoto et al., 2000; Cheung et al., 2007), highlight its usefulness for mechanistic studies in the process of traumatic TMJ ankylosis.

In this study, based on the animal model, we found an up-regulated expression of several members of Wnt signaling during the development of bony ankylosis, which was consistent with the expression changes of the bone-related markers. In addition, the gene expression profiles were related to the tissue composition of bony ankylosed callus according to the histological stages. Our results suggested that Wnt signaling was involved in the whole course of traumatic TMJ bony ankylosis.

Endochondral and intramembranous ossification are the two different types of new bone formation, not only under physiological but also pathological circumstances. The concept that similar pathways, for example BMP and Wnt signaling, may involved in different cartilage and bone pathology, especially regarding new bone formation, are supported by increasing evidences (Lories et al., 2009). For fracture healing and bone regeneration which are considered to recapitulate the process of embryonic skeletal development, the essential role of BMPs (Spector et al., 2001; Cho et al., 2002; Rosen, 2009) and Wnt signaling (Chen et al., 2007; Kim et al., 2007; Leucht et al., 2008) have been well identified. For osteoarthritis, a joint disorder characterized by cartilage degeneration and osteophyte formation, the activated BMP signaling and canonical Wnt signaling have been shown to promote the osteophyte formation through endochondral ossification process (van Beuningen et al., 1998; Zhu et al., 2009). For ankylosing spondylitis, BMP family members are critical in the early stages of the condition (Lories et al., 2005; Lories, 2011), and canonical Wnt signaling stimulates the progression of endochondral ossification (Lories et al., 2009; Schett and Rudwaleit, 2010; Braem and Lories, 2012). In this study, we provided preliminary evidences for the potential role of Wnt signaling in the development of traumatic TMJ bony ankylosis, which indicated the condition mimicked predominantly endochondral ossification process similar to the bone regeneration and the other skeletal diseases.

The TMJ is a specialized and complex structure that is only found in mammals. The formation of TMJ depends on two distinct mesenchymal condensations, which is different significantly from other synovial joints that develop by the cleavage or segmentation within a single skeletal condensation (Wang et al., 2011). Although the condyle and glenoid fossa are both derived from the cranial neural crest cells (Gu et al., 2008), the types of bone formation are distinct with the condyle undergoing endochondral ossification whereas the glenoid fossa being intramembranous ossification (Wang et al., 2011). Therefore, during the development of TMJ bony ankylosis, the bony fusion between the two traumatic articular surfaces has two characteristics different from the fracture healing of long bone. One is the difference in embryonic origin since the appendicular skeleton is derived from mesoderm. The other lies in the types of bone formation in the embryonic stage because for fracture healing, the fragment ends are the components of a certain skeleton thus possessing the same ossification pattern, whereas the condyle and glenoid fossa develop with different ossification pattern. So one might wonder whether the signaling pathways involved in the bony ankylosis and fracture healing are similar or not, and even whether the role of the signal molecules played in the two processes are similar or not.

In agreement with the study of mandibular bone regeneration in a mouse model by Leucht et al. (2008), we found an up-regulated expression of signal molecules of Wnt family in the TMJ bony ankylosis, indicating a similar signaling pathway was utilized in the condition despite the different embryologic origin and the nature of bone formation of the two processes (Yan et al., 2012a,b). Therefore, during the development of TMJ bony ankylosis, the bony fusion between the two traumatic articular surfaces has two characteristics different from the fracture healing of long bone. One is the difference in embryonic origin since the appendicular skeleton is derived from mesoderm. The other lies in the types of bone formation in the embryonic stage because for fracture healing, the fragment ends are the components of a certain skeleton thus possessing the same ossification pattern, whereas the condyle and glenoid fossa develop with different ossification pattern. So one might wonder whether the signaling pathways involved in the bony ankylosis and fracture healing are similar or not, and even whether the role of the signal molecules played in the two processes are similar or not.

5. Conclusion

Our study has shown, for the first time, the temporal expression patterns for members of the Wnt signaling and a series of genes regulating cartilage formation, bone formation and endochondral ossification during the formation of traumatic TMJ bony ankylosis in a sheep model, which provided useful information for advanced understanding of the molecular pathogenesis of traumatic TMJ bony ankylosis. Our results suggest that Wnt signaling is involved in the formation of the condition, thus may be a potential therapeutical target for the treatment of the disease in the future.

Conflicts of interest statement

The authors indicate no potential conflicts of interest.

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