Sustained Inflammation Induces Degeneration of the Temporomandibular Joint

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Biological

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ABSTRACT

The temporomandibular joint (TMJ) undergoes degenerative changes among patients who suffer from arthritis, and yet the pathogenesis of TMJ osteoarthritis and rheumatoid arthritis is poorly understood. We hypothesized that sustained inflammation in the TMJ induces structural abnormalities, and accordingly characterized the disc and synovium in a novel model with double injections of complete Freund's adjuvant (CFA), using behavioral, morphological, cellular, and molecular assessments. Thirty-five days following double CFA injections in seven-week-old female Sprague-Dawley rats, the disc in the CFA-induced inflammation group demonstrated multiple degenerative changes, including marked thickening, opacity, and deformation. The discs in the CFA group further showed significantly greater wet and net weights, and elevated collagen, aggrecan, and total glycosaminoglycan contents. The synovium in the CFA-induced inflammation group showed marked infiltration of mononucleated cells and accumulated subsynovial adipose tissue. Both the disc and synovium had significantly higher iNOS and IL-1β mRNA expression than controls (saline injections). These findings are consistent with our hypothesis that sustained TMJ inflammation, even within the presently observed 35 days, may be a predisposing factor for structural abnormalities. Insight into TMJ inflammation and degeneration is anticipated to improve our understanding of the pathogenesis of TMJ arthritis and help design clinically relevant strategies for tissue engineering.

KEY WORDS: inflammation, TMJ, disc, synovium, collagen, degenerative disease.

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INTRODUCTION

Temporomandibular joint disorders (TMD) represent a heterogeneous cluster of diseases (Stohler, 1999; Stegenga, 2010; Sessle, 2011). Osteoarthritis and rheumatoid arthritis of the TMJ are severe, debilitating disorders that can paralyze the entire masticatory system (Dimitroulis, 2005). The TMJ disc is an unusual structure and provides congruence for otherwise incongruent articulating surfaces of condyle and the glenoid fossa (Detamore and Athanasiou, 2003; Alhadlaq *et al.,* 2004). Recently, there has been robust interest in the regeneration of TMJ structures, including the mandibular condyle and disc (Feinberg *et al*., 2001; Alhadlaq and Mao, 2003; Allen and Athanasiou, 2006; Mao *et al.,* 2006). However, the roles of the TMJ disc and synovium in arthritis are poorly understood and therefore of direct relevance to studies and/or clinical care of TMJ pathophysiology and tissue engineering.

Previous studies have observed structural changes in the disc and synovium in individuals with TMD. In arthritis, the TMJ disc undergoes morphological changes, including thickening, displacement, lengthening, and folding (Taskaya-Yilmaz and Ogutcen-Toller, 2001; Melchiorre *et al.,* 2003). In severe cases, the disc undergoes perforation (Kuribayashi *et al.,* 2008). Furthermore, induced inflammation upon unilateral bite raise in the TMJ leads to an increase in the expression of aggrecan and versican in the disc and condyle (Mao *et al*., 1998). Pro-inflammatory cytokines such as IL-1β and TNFα are elevated in the synovial fluid of TMD patients (Suzuki *et al.,* 2002; Lai *et al*., 2006; Ogura *et al.,* 2010). We previously found that 17β-estradiol aggravates acute inflammation in the TMJ synovium through the NF-κB pathway (Kou *et al.,* 2011). However, the effects of sustained inflammation on degeneration in the TMJ are poorly understood. Accordingly, the present study was designed for the comprehensive evaluation of structural, genetic, and matrix changes in the TMJ disc and synovium upon sustained inflammation.

Several approaches have been adopted to induce acute and chronic inflammation in the TMJ. Surgical disruption to physiological structures, including bite raise, severance of the bilaminar zone, or induced condyle hypermobility, leads to inflammatory changes and subchondral bone degeneration in the TMJ (Mao *et al.,* 1998; Sharawy *et al*., 2003; Yamaza *et al*., 2003; Embree *et al.,* 2011). TMJ inflammation can also result from injection of chemical irritants, including carrageenan (Denadai-Souza *et al*., 2009), ovalbumin (Habu *et al*., 2002), and complete Freund's adjuvant (CFA) (Kerins *et al.,* 2004; Wu *et al*., 2010; Kou *et al*., 2011). CFA is a solution of antigen emulsified in mineral oil and consists of inactivated and dried mycobacteria. CFA injection is simple and reproducibly induces TMJ inflammation, as shown in previous work by us and

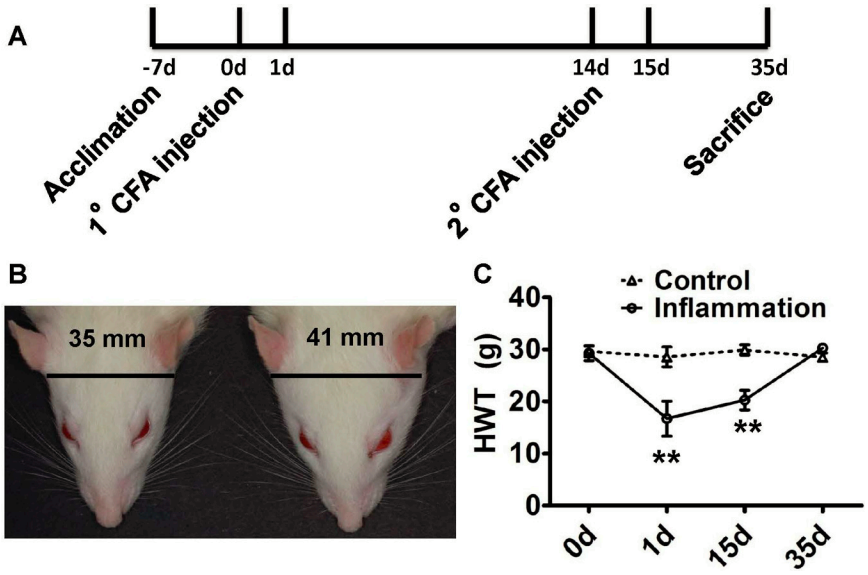

Figure 1. Time-course and behavioral assessment of induced chronic inflammation in the rat TMJ. **(A)** Time-course and procedures of induced chronic inflammation by injections of complete Freund's adjuvant (CFA) into the upper TMJ compartment. **(B)** Representative photographs one day after rats received saline injection (left) or CFA injection (right), and corresponding differences in head widths. Severe swelling was observed in the TMJ region one day after CFA injection. **(C)** The Head Withdrawal Threshold (HWT) was significantly lower one day after both the first and second CFA injections in the inflammation group compared with controls (mean ± SEM; N = 4; **p < 0.01).

others with a focus on the acute phase of inflammation (Harper *et al*., 2001; Guan *et al.,* 2005; Spears *et al.*, 2005; Flake *et al*., 2006; Lai *et al.,* 2006; Wu *et al*., 2010; Kou *et al.,* 2011). However, single-dose CFA injections in previous work were primarily associated with transient inflammatory changes in the TMJ. Furthermore, the majority of previous models have been characterized with isolated parameters (Harper *et al.,* 2001; Guan *et al.,* 2005; Spears *et al.,* 2005; Flake *et al.,* 2006; Lai *et al*., 2006) rather than complete assessments of morphological, behavioral, cellular, and molecular analyses. Here, we demonstrate that the TMJ synovium and disc undergo degenerative changes upon sustained inflammation of TMJ, with significant increases in iNOS and IL-1β, leading to thickened inflammatory discs with greater wet and net weights, and elevated collagen and aggrecan contents. These findings are consistent with our hypothesis that sustained inflammation is a predisposing factor for TMJ degeneration.

MATERIALS & METHODS

Induction of TMJ Inflammation

In total, 24 seven-week-old female Sprague-Dawley rats (wt, 180-200 g) were randomly assigned to the experimental or control group (N = 12/group). Inflammation was induced by a novel approach of double CFA injections (1:1 oil:saline emulsion, 50 μL; Sigma Aldrich, Shanghai, China) bilaterally into the upper compartment of the TMJ on Day 0 (1° injection) and Day 14 (2° injection) (Fig. 1A). Previous work has utilized only single CFA injections (Harper *et al*., 2001; Guan *et al.,* 2005; Spears *et al.,* 2005; Flake *et al.,* 2006; Lai *et al*., 2006). Rats in the control group received saline injections (same volume). All rats were housed under controlled temperature, on a 12-hour light/dark cycle with access to food and water. The experimental protocols were approved by the Animal Ethics Committee.

Measurement of Head Withdrawal Threshold *in vivo*

The head withdrawal threshold (HWT) was measured *per* our prior method (Wu *et al.,* 2010; Kou *et al*., 2011). Briefly, an electronic von Frey anesthesiometer (IITC Life Science, Woodland Hills, CA, USA) was used to record the smallest threshold force applied to the TMJ that elicited sudden head withdrawal. Four HWT measurements were taken: at baseline (Day 0 prior to injections), Day 1 and Day 15 (24 hrs following the 1°and 2° CFA injections, respectively), as well as Day 35 (immediately before the animals' death) (Fig. 1A).

Tissue Harvest and Measurement of Disc Thickness

All rats were euthanized by pentobarbital overdose 35 days after the first injection. The TMJ discs and synovia were harvested bilaterally from 6 rats *per* group for real-time quantitative PCR and histology. The bilateral TMJ discs from 6 other rats *per* group were harvested for weighing and biochemical assays. For histology, the TMJ disc, synovium, and subchondral bone were removed *en bloc*, fixed in 4% paraformaldehyde, and then demineralized in 15% EDTA. Paraffin-embedded specimens were sagittally cut in serial sections at 5-μm thickness and stained with hematoxylin and eosin. Two blinded examiners separately measured disc thickness for the anterior band, intermediate zone, and posterior band in 10 randomly selected sections *per* joint, with the averages pooled (N = 6/group). The longest linear distance was measured for the anterior and posterior bands, but the shortest distance was measured for the intermediate zone (Sun *et al*., 2009). The total number of mononucleated cells in each band (anterior, intermediate zone, and posterior) was counted (Image-Pro Plus v6.0, Media Cybernetics, Bethesda, MD, USA).

Statistical Analysis

Statistical analysis was performed with SPSS version 11.0. All data were presented as mean ± SEM. Following confirmation of normal data distribution, all data between the experimental and control groups were analyzed by Student *t* tests, with p values < 0.05 considered to be statistically significant.

RESULTS

Time-course of Inflammation and Behavioral Assessment

Intense swelling and redness over the TMJ region were observed 1 day after the first CFA injection, but not in the saline injection

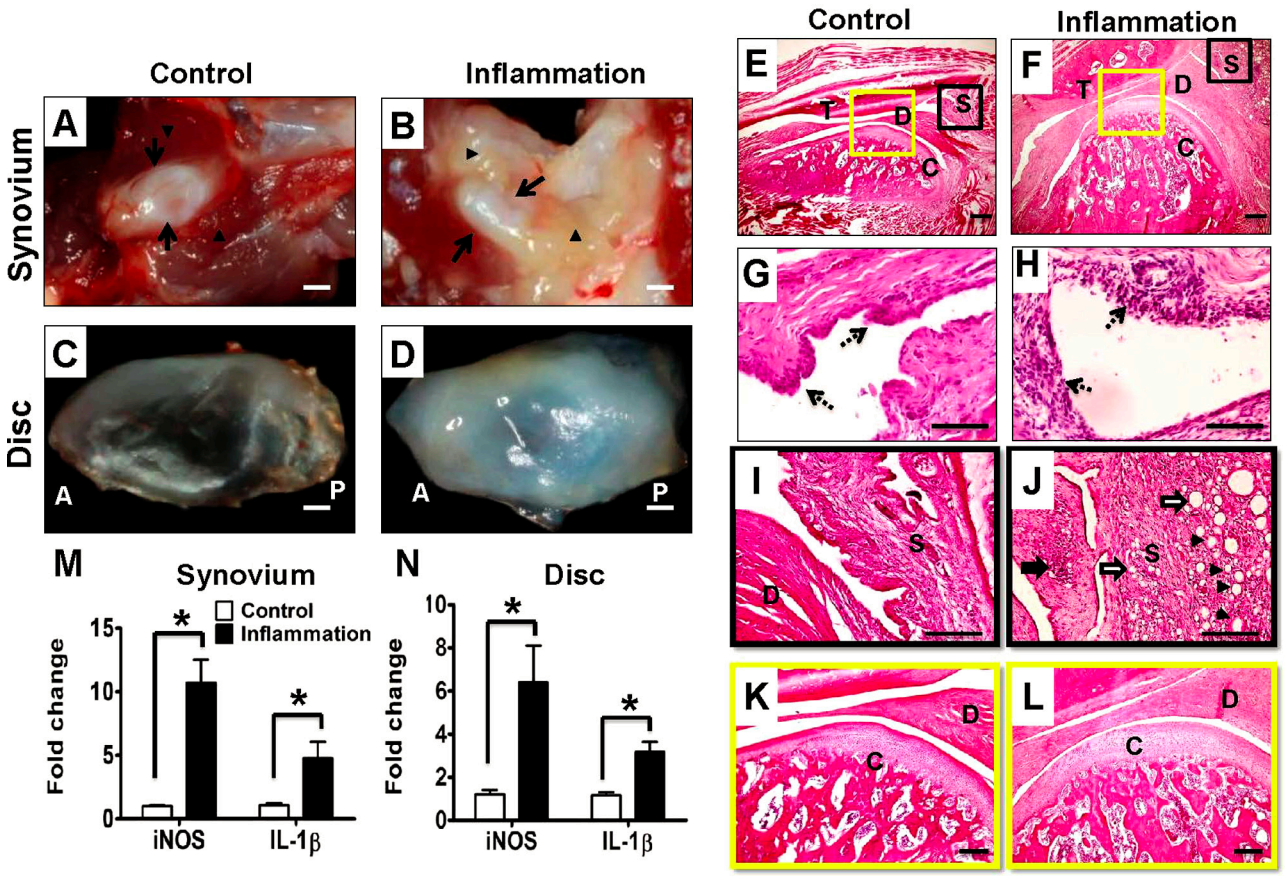

Figure 2. Histopathologic changes and mRNA expression of pro-inflammatory cytokines. **(A-D)** Representative morphological features of the synovium [(A,B) Arrow = disc; arrowhead = synovium; scale, 1 mm] and disc [(C,D) A = anterior; P = posterior; scale, 0.25 mm]. Both the synovium and disc became thickened and opaque in the inflammation group. **(E-K)** Representative microscopic features of the synovium and disc in the inflamed (right column) and control (left column) samples. (E, F) Representative microscopic sections of control and inflamed TMJ structures, respectively (T, temporal glenoid fossa; D, disc; S, synovium; C, mandibular condyle). The black and yellow squares in E are magnified in I and K, respectively. The black and yellow squares in F are magnified in J and L, respectively. Features characteristic of chronic synovitis are present in the inflammation group, including proliferation of synovial lining cells (H, dotted arrow), extensive infiltration of mononucleated, putative inflammatory cells in the subsynovial tissue (J, black arrow), proliferative and dilated blood vessels (J, arrowhead), and abundant lipid droplets (J, hollow arrow). These features are absent in controls. The representative inflamed TMJ disc sample showed deformation and thickening **(L)**, in comparison with the controls (K), with quantitative data shown in Figs. 3B and 3C. (Scale bar = 100 μm in G and H; scale bars = 200 μm in E, F, I, J, K, and L.) IL-1β and iNOS expression in the inflamed synovium **(M)** and disc **(N)** is significantly higher than that in controls by quantitative real-time PCR (N = 6; mean ± SEM; *$p < 0.05$).

group. The linear head width between bilateral TMJs showed marked increases in the inflammation group (Fig. 1B). Swelling gradually subsided within ~7 days following the first CFA injection, and showed no visual differences between experimental and control groups upon euthanasia at Day 35 (data not shown). HWT was virtually the same between two groups at baseline (0 d or immediately prior to the first CFA injections), but was significantly lower in the experimental group by Days 1 and 15, following the first and second CFA injections (Fig. 1C; $p < 0.01$; $N = 4$). HWT showed no significant differences between the two groups by the time of euthanasia (Fig. 1C). The time-course of HWT indicates that CFA-induced TMJ inflammation was likely acute following the first CFA injection, but subsided after the second CFA injection (Fig. 1C).

Cellular, Biochemical, and Molecular Changes

Sustained inflammation in the TMJ is a relative assessment. Previous work indicates that 14 to 28 days in small-animal models are sufficient to simulate sustained inflammation (Hutchins *et al.,* 2002; Sessle, 2011). Accordingly, we set 35 days as the endpoint for observing sustained inflammation following the first CFA injection. The second CFA injection (Day 14) was administered in consideration that TMJ inflammation may be exacerbated by repeated physical or chemical insults. Upon tissue harvest, the representative synovium in controls was thin and sufficiently transparent to reveal muscle color underneath (Fig. 2A). In contrast, the representative synovium in the experimental group was opaque and thickened (Fig. 2B). The representative control TMJ

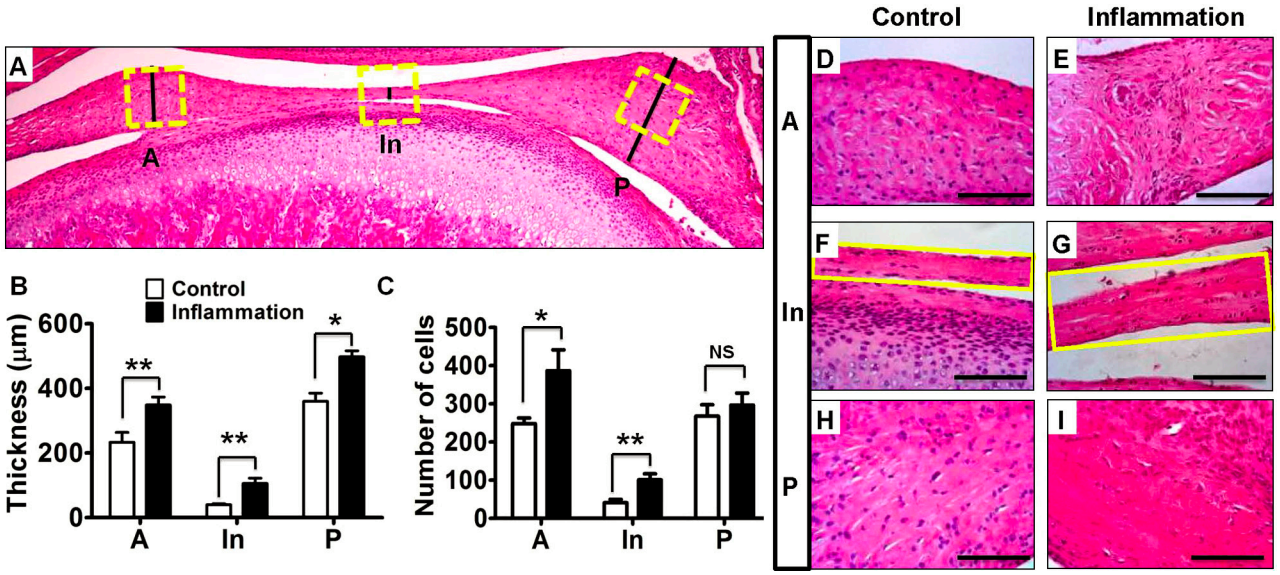

Figure 3. Increased thickness and cellularity in the TMJ disc upon induced chronic inflammation. **(A)** Photomicrograph showing the locations of disc thickness measurements and cell counting (A, anterior band; In, intermediate zone; P, posterior band). **(B)** Thickness of the disc in 3 bands was significantly higher upon induced chronic inflammation than in controls (N = 6; mean ± SEM; *p < 0.05, **p < 0.01). **(C)** The total number of mononucleated cells was significantly higher in the anterior band and intermediate zone upon induced chronic TMJ inflammation than in controls, but lacked statistical significance between control and inflammation groups for the posterior band (N = 6; mean ± SEM; *p < 0.05, **p < 0.01). Representative photographs of cellularity in the disc in control and inflamed samples **(D-I).** A, anterior; In, intermediate; P, Posterior. Scale: 100 μm.

disc was largely transparent and thin (Fig. 2C), showing the typical anterior band, intermediate zone, and posterior band (Fig. 2E). In contrast, the inflamed TMJ disc was thickened and deformed (Fig. 2D) and showed several characteristic changes (Fig. 2F). There were marked proliferation of synovial lining cells (Fig. 2H), apparent infiltration of mononucleated cells under the synovial membrane, proliferative and dilated blood vessels, and abundant sub-synovial lipid droplets in the inflamed synovium (Fig. 2J) in contrast to that in the controls (Figs. 2G, 2I). The representative inflamed disc, especially the intermediate zone, was thickened (Fig. 2L), in comparison with that in the controls (Fig. 2K; quantitative data in Figs. 3B, 3C). Few inflammatory or degenerative changes were observed in the mandibular condyle, likely because the irritant, complete Freund's adjuvant, was injected only into the upper TMJ compartment, instead of the lower compartment, which houses the mandibular condyle.

IL-1β and iNOS are two pro-inflammatory cytokines in sustained joint inflammation (Suzuki *et al.,* 2002; Takahashi *et al.,* 2003; Sessle, 2011), and are highly expressed in acute inflammation of the TMJ synovium (Kou *et al*., 2011). In the present work, iNOS and IL-1β expression, as quantified by real-time qPCR, was significantly elevated in the inflamed synovia ($p < 0.05$; N = 6; ~ 10-fold and ~ 5-fold, respectively, in Fig. 2M). In the inflamed TMJ disc, iNOS and IL-1β expression was also significantly elevated ($p < 0.05$; N = 6; ~ 6-fold and ~ 3-fold, respectively, in Fig. 2N). IL-1β and iNOS were immunolocalized in control and inflamed TMJ synovium and disc samples *per* our prior methods (Mao *et al*., 1998; Kou *et al*., 2011). CFA-treated TMJ disc and synovium samples showed marked IL-1β and iNOS expression, in comparison with control specimens (Appendix Fig.).

Gross Degenerative Changes

We quantitatively measured the thickness and weight of the TMJ discs. In controls, the TMJ discs were generally biconcave, thinnest in the intermediate zone (Fig. 3A). Upon induced inflammation, the disc became significantly thicker in all 3 bands (Fig. 3B; $p < 0.01$ for the anterior and intermediate bands; $p < 0.05$ for the posterior band; N = 6). The total number of cells in the inflamed group was significantly higher in the thickened anterior band ($p < 0.05$) and intermediate zone ($p < 0.01$) than in controls, but showed no significant differences in the posterior band ($p > 0.05$; N = 6) (Fig. 3C). Representative photomicrographs of controls and inflamed discs are shown in Figs. 3D-3I. The inflamed disc was significantly heavier in both wet and net weights than in controls (Fig. 4A; $p < 0.05$; N = 12).

Elevated Collagen and GAG Contents

The net collagen and GAG contents were significantly higher in the inflammation group than in controls (Fig. 4B; $p < 0.05$; N = 6). The relative collagen content over total disc weight was also significantly higher in the inflammation group (Fig. 4C; $p < 0.05$; N = 6). However, there were no significant differences in the relative GAG proportion (Fig. 4C; $p > 0.05$; N = 6), with standard collagen (HYP) and GAG regression curves shown in Figs. 4E and 4F, respectively. Real-time qPCR analysis showed

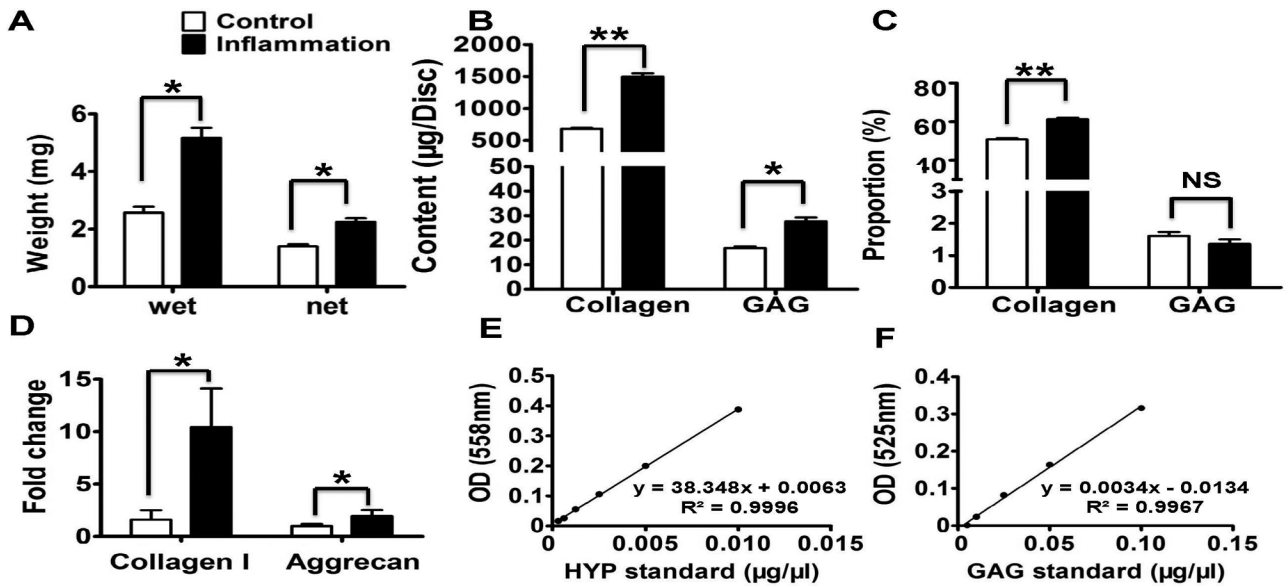

Figure 4. Increased weight and matrix macromolecules in the TMJ disc upon induced chronic inflammation. **(A)** Both the wet and net weights of the disc with induced chronic inflammation were significantly higher than in controls (N = 12). **(B)** The net collagen and GAG contents *per* disc were significantly higher in the inflamed discs than in controls. **(C)** The proportion of collagen in the inflamed group was significantly higher than in controls, but proportional GAG contents lacked significant differences between control and inflammation groups. **(D)** Collagen type I and aggrecan gene expression was significantly higher in inflamed discs than in controls. **(E, F)** Standard curves of biochemical analyses for HYP and GAG. (N = 6; mean ± SEM; *p < 0.05; **p < 0.01.)

that collagen type I and aggrecan expression was significantly higher in the inflammation group than in controls (Fig. 4D; $p < 0.05$; N = 6).

DISCUSSION

The primary discovery was that TMJ synovium and disc undergo degenerative changes following sustained inflammation, based on an experimental design with 3 areas of improvement over previous work. First, double CFA injections, for the first time, were shown to induce reproducible, sustained inflammation. Second, comprehensive analyses characterized a model that may be used broadly for TMJ studies. Third, sustained inflammation yielded aberrant structural, cellular, and molecular changes in TMJ disc and synovium. These degenerative changes included deformation, thickening, increases in wet and net weights, increased cellularity, increased iNOS and IL-1β expression, and significant increases of collagen type I and aggrecan contents, in addition to infiltration of mononucleated cells and abundant adipose tissue in the synovium. Aberrant changes in the structure of extracellular matrix and associated alterations of disc mechanical properties can surpass the adaptive capacity of disc and lead to clinically observed perforation (Kuribayashi *et al.,* 2008; Stegenga, 2010). Interestingly, head withdrawal threshold lacked any significant differences between the CFA and control groups after 35 days, suggesting that pain did not chronically persist. Thus, the observed structural deterioration may be independent of pain-induced activities such as altered muscle contraction patterns.

The most striking increases in cellularity in the TMJ disc were in the anterior and intermediate bands, which readily experience compressive loading during mastication. Previously, TMJ cell proliferation had been associated with sustained inflammation (Takahashi *et al.,* 2003; Kristensen *et al.,* 2008). Besides cellularity, increases in disc thickness may be accounted for by matrix characteristics. Type I collagen is the predominant component of the extracellular matrix in the TMJ disc in humans and rats (Kalpakci *et al*., 2011). GAG binds to type I collagen in the disc and serves to accumulate tissue fluid (Allen and Athanasiou, 2006). The increased collagen content may be accounted for by the increased cellularity and is likely a hypertrophic response (Abubaker *et al*., 1996; Okazaki *et al*., 1996). CFA has been injected into the superior joint compartment in the present and other studies (Harper *et al*., 2001; Guan *et al.,* 2005; Spears *et al.,* 2005; Flake *et a*l., 2006). In the present study, we observed few phenotypic changes in condylar cartilage. In contrast, osteoarthritic changes have been reported to take place in condylar cartilage and subchondral bone, including clefts and erosions, when CFA is injected into the inferior joint compartment (Cledes *et al*., 2006; Kuroki *et al*., 2011). Comparison of temporal phenotypes of double CFA injections in the superior and inferior compartments is warranted.

Sustained inflammation is characterized by up-regulation of pro-inflammatory cytokines. In this work, IL-1β and iNOS were up-regulated, suggesting that sustained inflammation in the TMJ is global, not only in the synovium, which is rich in vascular supply, but also in the avascular disc, similar to patients with TMJ arthritis (Suzuki *et al*., 2002). IL-1β and iNOS promote the

synthesis of type I collagen in chondrocytes and fibroblasts (Elias *et al*., 1990; Lertchirakarn *et al.,* 1998), which may partially account for the elevated collagen content. Although it is impossible to couple gross morphological changes directly to molecular events, we demonstrate that the HWT, a common behavioral assessment for the TMJ, shares a time-course similar to that of swelling and matrix changes in the inflamed TMJ disc and synovium. HWT is inversely associated with TMJ inflammation and pain (Ren, 1999), and is directly related to CFA injections. Although induced TMJ inflammation may decrease after ~14 days, inflammatory mediators persist for up to 6 wks (Kuroki *et al.,* 2011). The repeated intra-articular CFA administration in the present work is based on our pilot experiments in which inflammatory reactions upon single injection were insufficient to induce sustained inflammation. In short, analysis of the present data is consistent with our hypothesis that sustained inflammation may be a predisposing factor for TMJ degeneration. To date, few tissue engineering studies of the TMJ have been performed in inflamed TMJ models. Improved understanding of TMJ degeneration and inflammation will enrich our knowledge of pathogenesis of TMJ arthritis and help design clinically relevant strategies for tissue engineering.

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