Is It Possible to Withdraw Biologics From Therapy in Rheumatoid Arthritis?

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ABSTRACT

Background: Biologic agents targeting tumor necrosis factor (TNF) have revolutionized the treatment of rheumatoid arthritis (RA). Clinical remission is perceived as a realistic primary goal, and its maintenance leads to structural and functional remission.

Objective: This study reviews whether discontinuation of biologic agents is possible after sustained remission and discusses its significance from the risk/benefit point of view (including safety and health economic considerations).

Methods: Using a strategic PubMed search, 45 original research articles regarding discontinuation of biologic agents were identified; 7 were selected that had an obvious focus on discontinuation of biologic agents. These articles included the TNF20, BeSt (Behandel Strategieen), and RRR (Remission Induction by Remicade in RA) studies. However, because of the limitations of the original search, we also review here some articles that did not focus mainly on discontinuation of biologic agents but that presented data regarding biologic-free control. These studies included OPTIMA (Optimal Protocol for Treatment Initiation With MTX and Adalimumab), PRESERVE, and CERTAIN, as well as some recent findings in the HONOR (Humira Discontinuation Without Functional and Radiographic Damage Progression Following Sustained Remission) study from our department.

Results: In BeSt and OPTIMA, clinical remission was sustained without functional progression by discontinuing TNF inhibitors, after reducing disease activity by using TNF inhibitors and methotrexate (MTX), in patients with early RA and who were MTX naive. In some studies (including RRR and HONOR), the discontinuation of TNF inhibitors after sustained remission was possible in some patients with long-standing RA who had an inadequate response to MTX. When disease activity flared up after treatment discontinuation, re-treatment with infliximab or adalimumab was highly effective and safe in the majority of patients. It is also clear that tight control with TNF inhibitors and MTX seems to be a prerequisite for having a better chance of biologic-free remission.

Conclusions: Intensive treatment with TNF inhibitors may change the disease process of RA and potentially offers the possibility of a "treatment holiday" from biologic agents. (*Clin Ther.* 2013;35:2028–2035)

Key words: biologic, discontinuation, remission, rheumatoid arthritis, treatment.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by inflammation and joint destruction that causes significant morbidity and mortality. To prevent joint damage, disease-modifying antirheumatic drugs (DMARDs) such as methotrexate (MTX) should often be started after patients are diagnosed. However, the use of MTX monotherapy often fails to control disease activity and to prevent structural damage, and more effective treatment strategies are thus needed. TNF plays a pivotal role in the pathologic processes of RA through the accumulation of inflammatory cells and the self-perpetuation of inflammation, which leads to joint destruction. The combination of MTX and biologic agents targeting tumor necrosis factor (TNF) has revolutionized the treatment of RA, producing significant improvements in clinical, radiographic, and functional outcomes that were not previously observe. The combination has produced the emerging outcome and upcoming end

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point for the treatment as the followings.[1-5] Clinical remission is perceived as an appropriate and realistic primary goal in many patients, and its maintenance leads to structural and functional remission.

The possibility of discontinuation of biologic agent treatment after achievement of remission or low disease activity must be considered because of the long-term safety issues found by inhibiting a particular cytokine and the economic burden associated with expensive biological products. The decision to discontinue synthetic DMARDs should be made with caution; such discontinuation results in twice as many flare-ups, difficulties in reintroducing remission, and a halt in damage.[6] However, similar studies are not available for the biologic agents it remains unclear whether treatment strategies with biologics targeting induction and/or maintenance of clinical remission can potentially lead to subsequent discontinuation of the TNF inhibitors. The goal of the present article was to determine if discontinuation of biologic agents targeting TNF is possible in RA patients, after obtaining low disease activity or clinical remission during certain periods of use with TNF inhibitors. The content is based on results of a systemic literature review as well as new information.

METHODS

A search of PubMed was conducted by using a search strategy that combined terms for *rheumatoid arthritis, biological agent*, and *discontinuation, discontinuing,* or *cessation*. The systematic literature search strategy was as follows: #1, arthritis, rheumatoid [MeSH]; #2, biological agents OR biologics OR TNF inhibitor OR infliximab OR etanercet OR golimumab OR abatacept OR tocilizumab OR certolizumab pegol; #3, clinical trial [Filter]; #4, English [Filter]; #5, discontinuation OR discontinuing OR cessation; #6, review [Filter]; #7, juvenile idiopathic arthritis; and #8, #1 AND #2 AND #3 AND #4 AND #5 NOT #6 NOT #7.

The titles and abstracts of the citations were screened, and relevant articles were retrieved. The following selection criteria were used: (1) clinical trials of biologic agents in patients with RA, followed by discontinuation of the biologic agents due to preferable effectiveness but not to adverse events or to insufficient efficacy; (2) patients with RA aged >18 years; and (3) data available on 1 or more of the following prespecified outcomes: ratio of remission or low disease activity after at least 12 weeks of discontinuation or ratio of re-administration of the biologic agents.

Forty-five original research articles were identified from the PubMed search; 7 articles were selected as candidate studies, and 38 articles were excluded from our analysis. All of the included and excluded articles were published since 1998. The reasons for exclusion were categorized into 3 groups: (1) no description of discontinuing biologic agents; (2) reasons for discontinuing biologic agents were not specified; and (3) no description of discontinuing biologic agents due to preferable effectiveness. Characteristics of the candidate studies are summarized in the Table.[7-13]

The majority of the excluded articles focused on the efficacy and safety of certain biologic or synthetic DMARDs but not on discontinuation after attaining preferable disease control. The 7 included articles focused on discontinuation of biologic agents. However, those studies were published from a limited number of nations or institutes, and 4 were subanalyses of the BeSt (Behandel Strategieen) study and 2 were subanalyses of the RRR (Remission Induction by Remicade in RA) study. In addition, published evidence regarding biologic-free disease control is limited in cases of infliximab. We therefore reviewed some articles that did not mainly focus on discontinuation of biologic agents but that included data regarding biologic-free control, including the OPTIMA (Optimal Protocol for Treatment Initiation With MTX and Adalimumab), PRESERVE, and CERTAIN studies. We also included recent findings from the HONOR (Humira Discontinuation Without Functional and Radiographic Damage Progression Following Sustained Remission) study from our department.

RESULTS

Can We Discontinue Infliximab?

Infliximab is an anti-TNF chimeric monoclonal antibody that was approved for the treatment of RA in 1999 in the United States and the European Union. The study regarding biologic-free treatment in RA patients was first reported by a British group as a TNF20 study.[9,14] Patients with early RA who had <12 months of symptoms were treated with a combination of infliximab and MTX. Patients who initiated treatment with infliximab and MTX achieved higher American College of Rheumatology 50% and 70% improvement

Table. Summary of the candidate studies for discontinuation of biologic agents in patients with rheumatoid arthritis.

Author	Study	Nation	Biologic	DMARD	Criteria	Observation Period	No. of Discontinuations	Failed or Restarted	Effect of Restarting Biologic
van den Broek et al[7]	BeSt	NL	IFX	MTX	DAS ≤2.4 at 6 mo	7.2 y (median)	104 (52%): all 77: initial IFX + MTX	48% restart, 17 mo (median)	84% DAS ≤2.4
Klarenbeek et al[8]	BeSt	NL	IFX	MTX	DAS ≤1.6 at ≥6 mo	5 y	115/508 (23%): drug-free	53/115 (46%) restart, 23 mo (median)	39/53 (74%) DAS ≤1.6
Bejarano et al[9]	TNF20	UK	IFX	MTX	No criteria (randomized)	8 y	10 discontinued (1 died,) 4/9 REM, 1/4 drug free	5/9 (56%) failed	NS
Tanaka et al[10]	RRR	JPN	IFX	MTX	DAS28 ≤3.2 at >24 wk	1 y	114 discontinued, 102 evaluated at 1 y	46/102 (45%) failed	NS
Nawata et al[11]	CS	JPN	IFX	MTX	DAS28 <2.6 at ≥24 wk	NS	5% (9/172)	No. and %: NS, 14 mo (mean)	NS
van der Kooij et al[12]	BeSt	NL	IFX	MTX	DAS ≤ 2.4 at ≥6 mo	2 y	56% (66/117) initial IFX 29% (19/67) delayed IFX	NS	NS
van der Bijl et al[13]	BeSt	NL	IFX	MTX	DAS ≤2.4 ≥6 mo	2 y	56% (67/120) median 9.9 mo	15% (10/67) restart, median 3.7 mo	NS

BeSt = Behandel Strategieen; CS = case series; DAS = Disease Activity Score; DMARD = disease-modifying antirheumatic drug(s); IFX = infliximab; JPN = Japan; MTX = methotrexate; NL = the Netherlands; REM = remission; RRR = Remission Induction by Remicade in RA; UK = United Kingdom.

responses than those initiating therapy with MTX and placebo. One year after stopping induction therapy, response was sustained in 70% of patients who received infliximab and MTX. A significant reduction in magnetic resonance imaging evidence of synovitis and erosions at 1 year was also observed.

In the Netherlands, the BeSt study was conducted to compare 4 treatment strategies in patients with early RA.[7,8,12,13,15] Patients with disease duration <2 years after onset and a disease duration of 0.8 year were enrolled. A total of 508 patients with high disease activity estimated by using the Disease Activity Score in 44 joints (DAS44) were assigned to 4 groups and were evaluated by using the DAS44 every 3 months. DAS44 is a clinical assessment tool to integrate measures of disease activity which consists of swollen joint count and tender joint count of 44 joints, patient-evaluated global disease activity and CRP or ESR. Ninety (75%) of 120 patients in the fourth group who started treatment with infliximab achieved low disease activity, as shown by a DAS44 score ≤2.4; in 77 patients, infliximab was withdrawn because a DAS44 score ≤2.4 was maintained for 6 months. Low disease activity was maintained and progress of joint damage was inhibited in 67 patients who were treated with MTX monotherapy for 2 years after infliximab withdrawal. Furthermore, 5 years after receiving infliximab and MTX as initial treatment for RA, 58% of 120 patients had discontinued infliximab and 19% of patients had discontinued all DMARDs and remained in clinical remission or low disease activity, with minimal joint damage progression.

We initially conducted a multicenter prospective study (RRR) focused on the possibility of biologic-free remission in RA patients whose mean disease duration was 5.9 years.[4,5,10,11] This study included 114 patients with RA who reached and maintained low disease activity for >24 weeks with infliximab treatment. Among the 102 evaluable patients who completed the study, 56 maintained low disease activity after 1 year and showed no progression in radiologic damage and functional disturbance. The mean disease duration of the RRR achieved group was 4.8 (5.9) years, which made this study the first to prove patients with longer disease duration. It is noteworthy that re-treatment with infliximab in 32 patients was effective, and the majority of patients reached low disease activity (DAS28 scores <3.2) within 24 weeks. Minimal adverse reactions at infusion of the agent were seen in 5 patients only at the first or second infusion.

Can We Discontinue Etanercept?

Etanercept is a fusion protein of the TNF receptor and Fc of immunoglobulin G approved for RA treatment in 1998 in the United States and the European Union. Both the efficacy and safety of etanercept are well established. The PRESERVE trial was undertaken to determine if low disease activity could be sustained with reduced doses or withdrawal of etanercept in patients with moderately active RA despite MTX therapy.[16] After treatment with 50 mg of etanercept plus MTX for 36 weeks, 604 patients were randomized equally to receive 50-mg etanercept plus MTX, 25-mg etanercept plus MTX, or placebo plus MTX. Fifty-two weeks after randomization, 50 or 25 mg of etanercept with MTX in patients with moderately active RA more effectively maintained low disease activity (82.6% and 79.1%, respectively) than MTX alone after withdrawal of etanercept; low disease activity was sustained with MTX alone in 42.6% of patients after discontinuing etanercept.

Can We Discontinue Adalimumab?

Adalimumab is a fully human anti-TNF monoclonal antibody approved for RA treatment in 2003 in the United States and the European Union. The OPTIMA study reported a significant advantage with initial treatment with adalimumab plus MTX versus placebo plus MTX for achieving improved disease activity and structural changes in patients with MTX-naive RA and a mean RA duration of 3.9 months.[17,18] Of the 466 RA patients treated with adalimumab and MTX for 24 weeks, 207 (44%) achieved a stable DAS28 score (low disease activity) and were re-randomized to receive placebo or adalimumab with MTX. At week 78, more patients with continuous adalimumab treatment maintained low disease activity (91%) or remission (86%) than did patients in the adalimumab-free treatment group (81%) or remission (66%). Thus, withdrawal of adalimumab was possible in 66% to 81% of patients with early RA after achieving low disease activity. However, continued use of adalimumab and MTX yields better benefits with respect to work productivity than discontinuation of adalimumab.

The withdrawal of adalimumab in patients with early RA (mean RA duration, 1.7 months) was also assessed in a German study (designated HIT HARD).[19] During the first 24 weeks, 172 patients were treated with adalimumab or placebo with MTX;

after week 24, both groups were treated with MTX alone for 24 weeks. During the induction phase, 47% of patients treated with MTX and adalimumab achieved DAS28/remission; at week 48, 44% of these patients were still in remission by 24 weeks of adalimumab-free treatment.

Our group has performed a study (HONOR) similar to RRR by using adalimumab to investigate whether a sustained remission is preserved after discontinuation of adalimumab in patients with RA and an inadequate response to MTX.[5,20,21] Among 197 patients with RA who initiated treatment with a combination of adalimumab and concomitant MTX, 75 acquired sustained remission for at least 24 weeks. Fifty-one of these patients agreed to discontinue adalimumab, but 1 was lost to follow-up. The mean disease duration and DAS28 score in 75 patients was 7.1 years and 5.1 at baseline, respectively. Twenty-nine (58.0%) of the 50 patients achieved adalimumab-free remission at the primary end point of 6 months after discontinuation. However, 21 patients (42%) failed to maintain adalimumab-free remission for 6 months. Twelve of those patients (24%) experienced disease exacerbation, defined as DAS28 score using an erythrocyte sedimentation rate (ESR) >3.2 within a 6-month adalimumab-free period. Nine patients agreed to increase the MTX dosage and/or re-start adalimumab at the exacerbation. Among 12 patients with disease exacerbation, 5 of 6 patients re-treated with adalimumab returned to at least low disease activity within 6 months. Restarting adalimumab due to relapse was not associated with any harmful effects. These results, taken together with the results of the RRR study, suggest that restarting TNF inhibitors seems to be effective and safe even after a treatment holiday.

Can We Discontinue Certolizumab Pegol?

Certolizumab pegol is a recombinant, humanized antibody Fab' fragment, with specificity for human TNF, conjugated to 40 kDa of polyethylene glycol. It was approved for the treatment of RA in 2009 in the United States and the European Union. The CERTAIN study was undertaken to evaluate the maintenance of remission after withdrawal of certolizumab pegol in patients with low to moderately active RA despite DMARD therapy.[22] After 24 weeks of double-blind treatment with certolizumab pegol or placebo, 18.8% of patients treated with certolizumab pegol experienced remission (based on the clinical disease activity index [CDAI]) at both weeks 20 and 24; they discontinued the randomized therapy but remained on conventional DMARD treatment. After discontinuation, CDAI-categorized remission or low disease activity was retained up to week 52 in 3 and 7 patients, respectively, of the 17 patients who previously received certolizumab pegol treatment. Median time to loss of CDAI-categorized remission was 42.5 days. These results indicate that most patients with long-standing RA were unable to maintain remission after discontinuing certolizumab pegol.

What Is Relevant to the Discontinuation of Biologic Agents?

Recent studies indicate that some patients could discontinue TNF inhibitors without clinical flare and functional impairment after reduction of disease activity to low levels or remission by TNF inhibitors such as infliximab and adalimumab in combination with MTX. Although there are limited studies, a treatment holiday of TNF inhibitors seems possible in patients with not only early RA but also long-established RA. However, among multiple TNF inhibitors, infliximab and adalimumab seem to have the better potential for discontinuation than certolizumab pegol or etanercept, as shown in the studies of TNF20, BeSt, HIT-HARD, and OPTIMA in early RA, and RRR, HONOR, PRESERVE, and CERTAIN in established RA.[7–22] A monoclonal antibody against TNF such as infliximab and adalimumab blocks the biologic functions of TNF via bindings to not only soluble TNF but also transmembrane TNF, whose binding induces complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity, and outside-to-inside signaling, which would induce apoptosis to their pathogenic cells bearing membrane-bound TNF.[23–25]

However, not all patients with remission maintained that status even after discontinuation of adalimumab and infliximab. In the RRR study, 55% of patients sustained infliximab-free low disease activity for 1 year.[5,10] According to multivariate analysis, DAS28 scores at RRR study entry had the most marked correlation with the maintenance of low disease activity for 1 year after the discontinuation. By logistic regression and a receiver-operating characteristic curve analysis, the cutoff point for achieving RRR outcome, keep DAS28 <3.2 without flare, at the

time of patient enrollment was a DAS28 score of 2.225. In fact, 71.4% of patients whose DAS28 score at study entry was <2.225 maintained low disease activity for 1 year, whereas only 32.6% of patients whose DAS28 score at RRR entry was 2.225 to 3.2 maintained low disease activity. This finding indicates that "deep remission" was required to maintain low disease activity for 1 year after discontinuation of infliximab.

In the HONOR study, 58% of patients sustained adalimumab-free remission at 6 months.[5,20,21] A logistic regression analysis found that a lower DAS28-ESR score at discontinuation was the most significant predictive factor for adalimumab-free remission for 6 months, and a receiver-operating characteristic curve analysis found that the cutoff value of DAS28-ESR at discontinuation was 2.16. The percentage of patients who retained sustained remission at 6 months was 78% in the patient group with DAS28-ESR scores ≤ 2.16 at study entry and 22% in the patient group with scores of $2.6>$ DAS28-ESR >2.16. These results indicate that deep remission through tight control of disease activity at the discontinuation of biologic agents seems to be a prerequisite for the successful treatment holiday; these findings are analogous to those from RRR.

Thus, we have to realize that "intensive treatment" with TNF inhibitors is possible for efficiently bringing about a treatment holiday, as deep remission was shown to be a major factor affecting the success of the discontinuation of TNF inhibitors in 2 Japanese studies.[10,20,21] Furthermore, in our institution, among 577 patients who were treated with infliximab, 88 patients became free of biologic agents. By multivariate analysis, shorter disease duration and being negative for rheumatoid factor at the discontinuation of infliximab were found to most affect infliximab-free remission (data not shown, unpublished). Interestingly, 48% of the infliximab-free patients were negative for rheumatoid factor when infliximab was discontinued, although 77% of them were positive for it when infliximab was initiated.

It is important but difficult to determine how long preferable disease control can be sustained after discontinuing TNF inhibitors. Longitudinal observations as noted in the BeSt, TNF20, and RRR studies seem to offer some insight. In the BeSt study, the incidence of re-introduction of infliximab was reported, based on the number of risk factors according to the 8-year follow-up of infliximab-free survival in patients with early RA.[7,26] Ninety-four percent of patients who had no risk factors were sustained as infliximab-free. However, 42% of those who had 1 risk factor and 67% of those who had ≥ 2 risk factors needed to restart infliximab therapy. Only 2 of those who were nonsmokers and negative for anticitrullinated protein antibodies had the short treatment duration needed to re-introduce infliximab. Overall, $>50\%$ of all patients who discontinued infliximab successfully maintained DAS scores ≤ 2.4 for >8 years. In the 8-year follow-up of the TNF20 study in which patients with very early RA were enrolled, disease activity was significantly lower in the infliximab/MTX group than in the placebo/MTX group (median DAS28 score, 2.7 vs 4.3; $P = 0.02$).[9] Furthermore, 4 of 18 patients in the infliximab/MTX group kept DAS28 scores ≤ 2.6 and 1 patient achieved drug-free remission, whereas none of the placebo/MTX group remained in remission. In the RRR study, 29 of 104 patients had disease flares within 1 year (mean duration, 6.4 months) after the discontinuation of infliximab. By the 3-year follow-up, $\sim 70\%$ of patients failed to sustain low disease activity for 3 years after discontinuation.[26]

An advantage of a treatment holiday may be its cost-effectiveness, which includes the expected cost savings as well as quality-adjusted life-years. The BeSt study revealed that the best cost-effectiveness was observed in patients who initialized treatment with the combination therapy of MTX and infliximab among 4 treatment strategies.[27] The study found that longer quality-adjusted life-years resulted in better cost-effectiveness from both a societal and a health care perspective. This finding might be due to improved productivity that almost completely compensated for the extra medication cost as well as an increase in successful discontinuation of infliximab.

Data from animal arthritis studies indicate that the knockout mutation of TNF gene in these models reveals the amelioration of both the incidence and severity of the arthritis and that TNF is pivotally involved in the process of the disease. Because biologic agents targeting TNF substantially reduce the protein levels of TNF in the body, TNF gene–targeting models offer education on the pleiotropic bioactivity of TNF. Thus, if animal data partially reflect the efficacy of TNF inhibitors in patients with RA, it is implied that TNF inhibitors may change "the course of the disease" or induce "immunologic remission." The higher

incidence of seroconversion of rheumatoid factor in patients who successfully discontinued TNF inhibitors might reflect changes in immunity in patients with RA. Thus, although the studies are limited, some patients with RA may achieve a treatment holiday from TNF inhibitors, when disease course is successfully changed by intensive treatments with MTX and TNF inhibitors.

CONCLUSIONS

After the sustained remission by biologic agents targeting TNF in MTX-naive RA patients and RA patients with inadequate response to MTX, discontinuation of biologic agents is emerging from the risk/benefit point of view, including safety and economical issues. After discontinuing TNF inhibitors, patients with RA could successfully remain in low disease activity or remission without radiologic and functional damage progression of articular destruction. Such a discontinuation of TNF inhibitors is possible not only in early and active RA but also in some patients with longstanding RA. However, "deep remission," which is supposedly induced by intensive treatment using the combination of MTX and TNF inhibitors, is a prerequisite to obtain a better chance of a treatment holiday.

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CONFLICTS OF INTEREST

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IL-17 Inhibits Chondrogenic Differentiation of Human Mesenchymal Stem Cells

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Abstract

Objective: Mesenchymal stem cells (MSCs) can differentiate into cells of mesenchymal lineages, such as osteoblasts and chondrocytes. Here we investigated the effects of IL-17, a key cytokine in chronic inflammation, on chondrogenic differentiation of human MSCs.

Methods: Human bone marrow MSCs were pellet cultured in chondrogenic induction medium containing TGF-β3. Chondrogenic differentiation was detected by cartilage matrix accumulation and chondrogenic marker gene expression.

Results: Over-expression of cartilage matrix and chondrogenic marker genes was noted in chondrogenic cultures, but was inhibited by IL-17 in a dose-dependent manner. Expression and phosphorylation of SOX9, the master transcription factor for chondrogenesis, were induced within 2 days and phosphorylated SOX9 was stably maintained until day 21. IL-17 did not alter total SOX9 expression, but significantly suppressed SOX9 phosphorylation in a dose-dependent manner. At day 7, IL-17 also suppressed the activity of cAMP-dependent protein kinase A (PKA), which is known to phosphorylate SOX9. H89, a selective PKA inhibitor, also suppressed SOX9 phosphorylation, expression of chondrogenic markers and cartilage matrix, and also decreased chondrogenesis.

Conclusions: IL-17 inhibited chondrogenesis of human MSCs through the suppression of PKA activity and SOX9 phosphorylation. These results suggest that chondrogenic differentiation of MSCs can be inhibited by a mechanism triggered by IL-17 under chronic inflammation.

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Introduction

Chondrocytes were considered as the only cell type that exists in the articular cartilage until recently. Due to the limited regenerative ability of chondrocytes, cartilage defects in patients with rheumatoid arthritis (RA), osteoarthritis (OA), and trauma are irreversible and cartilage repair is considered difficult. However, some reports have characterized mesenchymal stem cells (MSCs) in the articular cartilage as chondrocyte progenitor cells [1–3]. MSCs are multipotent cells capable of differentiation into osteoblasts and chondrocytes, and can be easily obtained from mesodermal tissues, such as bone marrow and adipose tissue [4]. We reported previously that MSCs can effectively differentiate into osteoblasts in the presence of IL-1β through the non-canonical WNT5A/ROR2 signaling pathway [5]. In addition, MSCs produce high amounts of osteoprotegerin, a decoy receptor for receptor activator of nuclear factor kappa-B ligand (RANKL), and efficiently suppressed osteoclast differentiation, highlighting the potential importance of MSCs in joint repair treatments [6]. Furthermore, several studies have shown that implanted MSCs can differentiate into chondrocyte-like cells *in vivo* [7,8]. Given the finding that MSCs exist in articular cartilage, it is conceivable that MSCs contribute to the maintenance of cartilage homeostasis and, moreover, possess an intrinsic ability to repair cartilage defects. TNF-α has been reported to inhibit chondrogenic differentiation of human MSCs [9] and, correspondingly, the repair of articular cartilage can be observed in some RA patients treated with TNF inhibitors [10].

The relationship between chronic inflammation and bone or cartilage metabolism has been gaining attention in recent years. IL-17A (hereafter referred to as IL-17), a key player in chronic inflammation, is characterized as a bone metabolism-related

cytokine [11]. In animal models that have employed strategies to increase expression, neutralize activity, or delete IL-17, IL-17 plays a distinct pathogenic role in inflammatory arthritis [12–14]. In human studies, high concentrations of IL-17 have been found in the synovial fluid of patients with RA [15–17] and IL-17-producing $CD4^+$ T lymphocytes (Th17 cells) have been detected in RA synovial membranes [18]. In cartilage, IL-17 induces cartilage matrix breakdown by increasing the expression of matrix metalloproteinases (MMPs) in synoviocytes and chondrocytes [19,20], as well as apoptosis of chondrocytes, which is thought to contribute to cartilage destruction [21]. However, there are only few reports on the effects of inflammatory cytokines on chondrocyte differentiation. In particular, inflammatory cytokines are concentrated in the joints of RA patients and are predicted to inhibit chondrogenic differentiation of MSCs. However, the specific effects of IL-17 on chondrogenesis have not been reported.

Transforming growth factor beta (TGF-β) is a pleiotropic growth factor that is involved in multiple cellular processes and is widely known to promote chondrogenic differentiation [4]. Among the various TGF-β signaling pathways, SMADs, the downstream transcription factors, play a distinct role in regulation of cartilage related genes [22]. On the other hand, SRY-type HMG box9 (SOX9) has been identified as a master transcription factor in chondrogenic differentiation [23–25]. Transcriptional activity of SOX9 is regulated by not only its expression level but also several post-translational modifications including phosphorylation. Cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) is a key kinase known to phosphorylate SOX9 [26,27]. However, the effect of IL-17 on these TGF-SMAD pathway and PKA-SOX9 axis remains unclear.

Here we evaluated the effects of IL-17 on chondrogenic differentiation of human MSCs utilizing an *in vitro* pellet culture system with TGF-β3. The results showed that IL-17 inhibited chondrogenesis through a mechanism involving PKA and SOX9 activity. These findings have important implications for the design of clinically effective cartilage repair therapies.

Materials and Methods

Cell Culture

Human MSCs were purchased from Lonza (Walkersville, MD). Multipotency was confirmed by differentiation of MSCs into osteoblasts, chondrocytes, and adipocytes. Cell surface markers were positive for CD29, CD44, CD105, and CD166 and negative for CD14, CD34, and CD45. Cells were cultured following the instructions recommended by the manufacturer. Cells were cultured in MSC growth medium (MSCGM) (Lonza) at 37°C in a 5% CO_2 atmosphere and maintained at subconfluence to prevent spontaneous differentiation. Cells from passage 2–4 were used in this study.

Chondrogenesis and Cell Treatment

Human MSCs at subconfluent conditions were trypsinized and aliquots of 2×10^5 cells per well were added to an ultra-low attachment surface, round-bottom 96-well plate (Corning, New York, NY), and the plate was spun at $400\times g$ for 5 min. For differentiation into chondrocytes, cells were cultured in a commercialized chondrogenic induction medium (hMSC Differentiation BulletKit-chondrogenic, Lonza) in the absence or presence of 10 ng/mL recombinant human TGF-β3 (Lonza). The cell pellets formed free-floating aggregates within the first 24 h. To analyze the effects of inflammatory cytokines or a kinase inhibitor, recombinant human IL-17A (Pepro Tech EC, London, UK), TNF-α (R&D Systems, Minneapolis, MN), IL-1β (RELIATech, San Pablo, CA), or the PKA inhibitor H89 (Enzo Life Sciences, Plymouth Meeting, PA) were added to the culture medium during chondrogenic induction. The medium was replaced every 2–3 days, and aggregates were collected at the indicated time points for analysis.

Histology and Immunohistochemical Staining

Aggregates were harvested 21 days after chondrogenic induction, fixed for 3 h in 10% buffered formalin at room temperature, and prepared for paraffin embedding. To detect matrix proteoglycans, sections (4 μm thickness) were stained with 0.1% Safranin O solution (Muto Pure Chemicals, Tokyo, Japan) for 2 min and counter-stained with hematoxylin. For immunohistochemistry, sections were deparaffinized, hydrated, and incubated in 0.4 mg/mL proteinase K (Dako, Glostrup, Denmark) for 5 min. Endogenous peroxidases were quenched in 3% hydrogen peroxide solution (Wako Pure Chemicals, Osaka, Japan) for 30 min. After washing with phosphate buffered saline (PBS) and incubation in blocking solution (ProteinBlock, Dako) for 1 h, the slides were incubated with polyclonal rabbit anti-human type II collagen antibody (ab34712, Abcam, Cambridge, MA) at a 1:200 dilution for 1 h at room temperature. Sections were then washed with PBS and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Nichirei, Tokyo, Japan) for 30 min. Antigens were visualized using a 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate (Dako) and counter-stained with hematoxylin. Slides were coverslipped and examined using BIOREVO BZ-9000 (Keyence, Osaka, Japan). A Plan Apo 10×/0.45 objective (Nikon, Tokyo, Japan) and BZ-II Viewer software (Keyence) were used for image acquisition and processing.

Measurement of Sulfated Glycosaminoglycan Content

Aggregates were harvested at day 21 and washed with PBS. Samples were homogenized using a microhomogenizer (Sarstedt, Nümbrecht, Germany) in digestion buffer [30 mM Tris (pH 7.8), 50 mM NaCl, and 10 mM $MgCl_2$] containing 100 μg/mL of proteinase K (Sigma, St. Louis, MO), then incubated overnight at 65°C. Sulfated glycosaminoglycan (sGAG) content in aggregate digests was quantified using the dimethylmethylene blue (DMMB) dye-binding assay (Blyscan, Biocolor, County Antrim, UK). In brief, the digests of aggregates were combined with DMMB solution, and sample absorbance was measured at 656 nm using a microplate reader. sGAG concentrations were calculated from a standard curve generated with bovine tracheal chondroitin 4-sulfate.

Gene Expression Analysis using Real-time Polymerase Chain Reaction (PCR)

Total RNA was extracted from each aggregate using a microhomogenizer (Sarstedt) in RLT buffer (Qiagen, Hilden, Germany). Total RNA was purified using a RNeasy mini kit (Qiagen) and first strand cDNA was prepared using the high capacity RNA-to-cDNA kit (Applied BioSystems, Foster City, CA) according to the specifications provided by the manufacturer. Real-time PCR was performed in a StepOne Plus system (Applied BioSystems). Gene expression was analyzed with TaqMan® Gene Expression Assay (Applied BioSystems) primer/probe pairs: β-actin (Hs99999903_m1), type II collagen (*COL2A1*, Hs00264051_m1), aggrecan (*ACAN*, Hs00153936_m1), type X collagen (*COL10A1*, Hs00166657_m1), alkaline phosphatase (*ALP*, Hs01029144_m1), and IL-17 receptor A (*IL-17RA*, Hs01064648_m1). The relative expression level of each gene was normalized to that of β-actin, and relative transcript quantities

were compared with an MSC control [cultured in conventional 2-dimensional (2D) conditions] and analyzed using the $2^{-\Delta\Delta Ct}$ method [28].

Western Blotting

To analyze the effect of IL-17 on SMAD2 expression, cells were seeded at a density of 1×10^4 cells/cm^2 on a 24-well plastic plate in DMEM containing 5% FCS and antibiotics. Thereafter, TGF-β3 (10 ng/mL) was administered after 16 h starvation in FCS-free medium. After 15 min incubation, cells were washed with ice cold PBS. IL-17 was added throughout the entire culture period. For the preparation of whole cell lysates, cells were lysed with lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, and 1% Nonidet P-40, supplemented with a protease and phosphatase inhibitor cocktail tablet (Complete Mini and PhosSTOP, respectively, Roche, Indianapolis, IN). Nuclear and cytoplasmic extracts were collected using the Affymetrix Nuclear Extraction Kit (Affymetrix, Fremont, CA). The procedure was carried out according to the manufacturer's protocol. Analysis of chondrogenic aggregates were performed with cell aggregates homogenized in lysis buffer after treatment with IL-17 or H89 for the indicated periods of time. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL) and equal amount of protein was loaded in each experiment. Immunoblotting was performed with antibodies against SMAD2 (D43B4, Cell Signaling Technology, Beverly, MA), phospho-SMAD2 (138D4, Cell Signaling Technology), SOX9 (ab26414, Abcam) or phospho-SOX9 (ab59252, Abcam) followed by appropriate secondary antibodies (GE Healthcare, Little Chalfont, UK). β-actin (A-1978, Sigma) and TATA box-binding protein (TBP) (ab818, Abcam) were used as loading controls. To quantify the band intensities, densitometric analyses were performed using a CS Analyzer, version 3.0 (Atto, Tokyo, Japan) image analysis software. The relative value of each band was calculated as the intensity of the target band divided by the intensity of the loading control.

Immunofluorescence Microscopy

For indirect immunofluorescence microscopy, cells cultured on cover-glass slides were fixed with cold MeOH for 15 min. Samples were subsequently treated with PBS containing 1% BSA for 30 min, and then incubated with the primary antibody (SMAD2: D43B4, Cell Signaling Technology) at a 1:50 dilution for 1 h at room temperature. Cells were washed with PBS and then incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:100 dilution for 30 min at room temperature. After washing the cells with PBS, samples were incubated with 1 μg/mL DAPI (Santa Cruz Biotechnology) and then mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Slides were examined using BIOREVO BZ-9000 (Keyence, Osaka, Japan). A Plan Fluor ELWD 20×/0.45 objective (Nikon) and BZ-II Viewer software (Keyence) were used for image acquisition and processing.

In vitro Kinase Assay for PKA Activity

PKA activity was measured using a PepTag® Assay for non-radioactive detection of PKA (Promega, Madison, WI), according to the instructions provided by the manufacturer. After 7 days of culture with IL-17 or H89, three aggregates from each group were pooled and homogenized using a microhomogenizer (Sarstedt) in 0.15 mL of cold PKA extraction buffer consisting of 25 mM Tris (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, and 10 mM β-mercaptoethanol, and was supplemented with a protease inhibitor cocktail tablet (Roche). After centrifugation of the homogenates (14,000×rpm, 5 min, 4°C), the supernatants were obtained and mixed with the other components. All reaction components were added on ice to a final volume of 25 μL of the following mixture: 5 μL 5×PKA reaction buffer, 5 μL PepTag®A1 Peptide (0.4 μg/μL), 5 μL 5×PKA activator, 1 μL Peptide Protection Solution, and 4 μL water. After mixing these reagents, 5 μL protein sample or PKA catalytic subunit (2 μg/mL, positive control) or water (negative control) were added and the mixtures were incubated for 30 min at room temperature. After terminating the reaction by heating the samples at 95°C for 10 min, the samples were loaded onto 0.8% agarose gel before electrophoresis. The phosphorylated peptide migrated toward the cathode while the non-phosphorylated peptide migrated toward the anode. Bands were visualized under UV light, and the intensity of each band was quantified as described above. The relative PKA activity was expressed as the ratio of band intensity compared with the intensity of the "no TGF-β3" sample.

Measurement of DNA Content

The DNA content of aggregates was determined using the Quanti-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA) according to the instructions provided by the manufacturer. After washing the aggregates with PBS, the aggregates were homogenized and sonicated in TE buffer (200 mM Tris HCl and 20 mM EDTA) containing 0.2% Triton X-100 on ice. Samples were assayed by mixing PicoGreen reagent and fluorescence was detected using a microplate fluorescence reader (Ex/Em = 485/535 nm).

Statistical Analysis

All quantitative data were expressed as mean ± standard deviation (SD). Differences between two groups were tested for statistical significance by the Student's unpaired two-tailed *t*-test. For comparison of more than three groups, analysis of variance (ANOVA) was used. If the ANOVA was significant, the Dunnett's multiple comparison test was used as a post hoc test. Statistical analyses were performed using GraphPad Prism version 4.00 (GraphPad, San Diego, CA). A P value <0.05 was considered significant.

Results

Suppressive Effect of IL-17 on Chondrogenic Differentiation of Human MSCs

Although IL-17, TNF-α and IL-1β are important inflammatory cytokines, the effect of IL-17 on chondrogenic differentiation has not been characterized. Therefore, we first estimated the effect of IL-17 on chondrogenesis of human MSCs. Human MSCs were pellet-cultured in a chondrogenic induction medium containing TGF-β3 or a non-inductive control medium that lacked TGF-β3. During culture in chondrogenic induction medium containing TGF-β3, the size of cell aggregate gradually increased owing to the accumulation of cartilage matrix proteins. Chondrogenic induction for 14 days resulted in enlarged aggregates with a spherical shape compared with the non-inductive control (Figure 1A). After 21 days of culture in TGF-β3-containing medium, a marked increase in cartilage matrix molecule, proteoglycan, and type II collagen expression was demonstrated by staining with Safranin O and anti-type II collagen antibody (Figure 1B). The addition of TNF-α or IL-1β to the chondrogenic induction medium containing TGF-β3 inhibited the increase in aggregate size (Figure 1A). Although IL-1β inhibited both proteoglycan and type II collagen accumulations, TNF-α mainly inhibited type II collagen accumulation with less effect on proteoglycan (Figure 1B).

The addition of IL-17 showed inhibitory effects on aggregate size and cartilage matrix accumulation in a dose-dependent manner. Although higher IL-17 concentration was necessary to induce inhibitory effect compared to IL-1β and TNF-α, apparent dose-dependency with intense effect at 100 ng/mL was observed (Figure 1A and B). To quantify the effect of IL-17, the wet weight and sGAG content of the aggregates were measured. IL-17 (at 10 or 100 ng/mL) significantly reduced the TGF-β3-dependent increase in weight and sGAG content in a dose-dependent manner (Figure 1C and 1D). Measurement of DNA content indicated that the inhibitory effect of IL-17 was not dependent on cell growth inhibition or induction of apoptosis (data not shown).

Next, we investigated the sequential expression of IL-17 receptor A (*IL-17RA*) during chondrogenic differentiation of human MSCs. Although the expression of *IL-17RA* was transient, it increased significantly (on day2 and 7), reaching a peak level at day 2 and gradually decreasing to baseline by day 14 (Figure 1E).

To better characterize the effect of IL-17, the expression of chondrogenic marker genes were measured. Type II collagen (*COL2A1*), aggrecan (*ACAN*), type X collagen (*COL10A1*), and alkaline phosphatase (*ALP*) were increased in the aggregate at day 14 and dose-dependently suppressed following the addition of IL-17 (Figure 2). Therefore, the suppressive effect of IL-17 on cartilage matrix accumulation observed in Figure 1 was considered to result from the inhibition of *de novo* cartilage matrix protein synthesis.

Suppressive Effect of IL-17 on SOX9 Phosphorylation, but not on SMAD2

TGF-β3 is an essential factor for chondrogenic differentiation of MSCs in our assay system. To evaluate the direct effect of IL-17 on TGF-β signal transduction pathway, we first evaluated the expression and phosphorylation of SMAD2, the main transducer of TGF-β signaling. Phosphorylation of SMAD2 was increased by TGF-β3 stimulation, and the addition of IL-17 did not affect either phospho-SMAD2 or total SMAD2 expression (Figure 3A). In order to confirm this effect, we analyzed the cytoplasmic and intranuclear SMAD2. TGF-β3 treatment induced a slight decrease of cytoplasmic SMAD2, whereas a prominent increase of the SMAD2 and phospho-SMAD2 was detected in the nuclear fraction proving its phosphorylation and nuclear translocation. Addition of IL-17 altered neither the expression nor phosphorylation of SMAD2 in both nucleus and cytoplasm (Figure 3B). This observation was visually confirmed by immunofluorescence microscopy analysis (Figure 3C). Next, we assessed the effect of IL-17 on SOX9, known as the master transcription factor for chondrogenesis [23–25] and its transcriptional activity is regulated by phosphorylation [26,27]. Total SOX9 expression was detectable in undifferentiated MSCs and increased during culture with chondrogenic medium supplemented with TGF-β3, reaching a peak level on day 4, and plateaued thereafter, albeit with a slight decline. In contrast, SOX9 phosphorylation was not observed in undifferentiated MSCs, but was strongly induced by chondrogenic medium supplemented with TGF-β3 by day 2 and was sustained through day 21 (Figure 3D). The addition of IL-17 did not alter total SOX9 or SOX9 phosphorylation on day2. However, IL-17

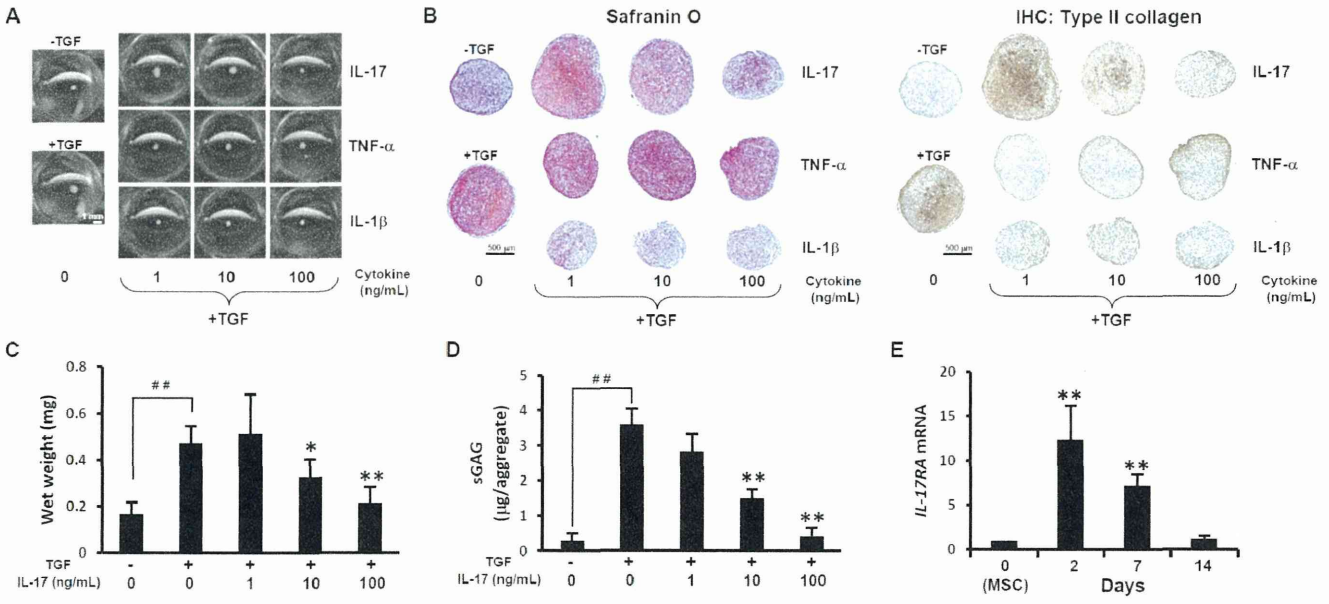

Figure 1. IL-17 inhibits TGF-β3-induced chondrogenic differentiation. A, Human mesenchymal stem cells (MSCs) were cultured in pellets, with or without TGF-β3 (10 ng/mL). Macro-images of the aggregates cultured with IL-17, TNF-α or IL-1β at the indicated concentrations for 14 days. Scale bar represents 1 mm. B, Aggregates cultured in the presence of the indicated cytokines for 21 days were fixed and paraffin embedded, then sections were stained with Safranin O and anti-type II collagen antibody. Original magnification×10. Scale bars represent 500 μm. (A and B) Data are representative of two independent experiments with similar findings. C, Wet weight of aggregates treated with IL-17 for 14 days. Values are mean±SD of 6–7 aggregates per group from three independent experiments with similar tendencies. $^{\#\#}P<0.01$, compared to no cytokine (−TGF-β3), by Student's *t*-test. $^{*}P<0.05$; $^{**}P<0.01$, compared to no cytokine (+TGF-β3), by Dunnett's multiple comparison test. D, Sulfated glycolaminoglycan (sGAG) content in aggregates treated with IL-17 for 21 days. Values are mean±SD of 3–4 aggregates per group from two independent experiments with similar findings. $^{\#\#}P<0.01$, compared to no cytokine (−TGF-β3), by Student's *t*-test. $^{**}P<0.01$, compared to no cytokine (+TGF-β3), by Dunnett's multiple comparison test. E, IL-17 receptor A (*IL-17RA*) mRNA levels in aggregates were determined by real-time PCR for the indicated time points. Values are normalized to β-actin expression and expressed as mean±SD of 6 aggregates per group from three independent experiments with similar tendencies. $^{**}P<0.01$, compared to day 0 (undifferentiated MSC), by Dunnett's multiple comparison test.
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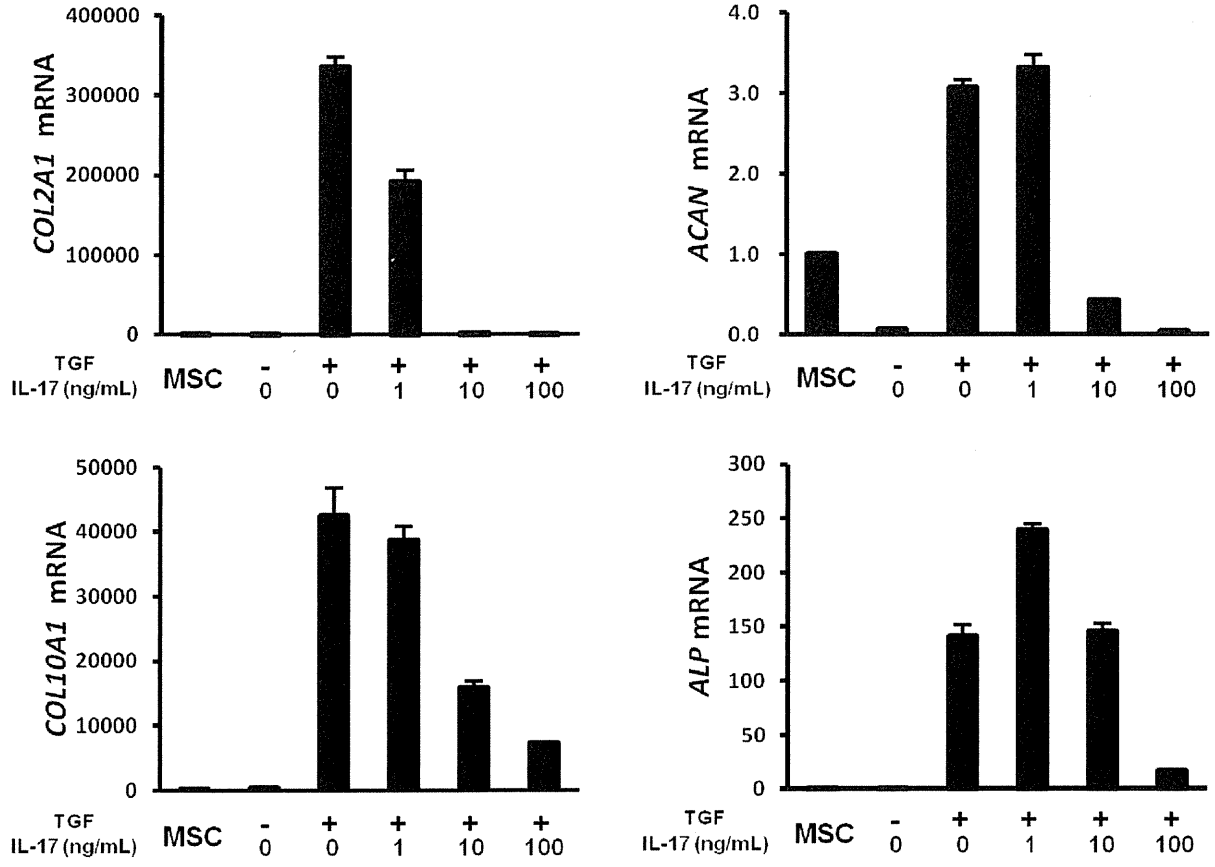

Figure 2. IL-17 suppressed the expression of chondrogenic marker genes. Human MSCs were cultured as aggregates in TGF-β3-containing medium with the indicated concentrations of IL-17. After 14 days, type II collagen (*COL2A1*), aggrecan (*ACAN*), type X collagen (*COL10A1*), and alkaline phosphatase (*ALP*) mRNA levels were determined by real-time PCR and expressed relative to β-actin expression level. Values are mean±SD of 3 aggregates from 1 of 3 independent experiments with similar findings.
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suppressed SOX9 phosphorylation in a dose-dependent manner on day7 without affecting total SOX9 expression (Figure 3E). Densitometric analysis indicated significant inhibition of phospho-SOX9 by IL-17 (at 100 ng/mL) on day7 (Figure 3F).

IL-17 Inhibits PKA Activity

PKA is a serine/threonine kinase that plays an important role in SOX9 phosphorylation [26]. Therefore, we next investigated the effect of IL-17 on PKA activity. Due to the significant inhibition of SOX9 phosphorylation on day7 (Figure 3F), PKA activity was evaluated on the same day. Enzymatic PKA activity in the aggregate was increased on day 7 of pellet culture in chondrogenic medium supplemented with TGF-β3 (Figure 4A). The addition of IL-17 (100 ng/mL) significantly decreased PKA activity. However, the latter effect of IL-17 was less than that of H89 (10 μM), the most frequently used selective PKA inhibitor (Figure 4A). In addition, we confirmed that inhibition of PKA activity by H89 resulted in the suppression of SOX9 phosphorylation (Figures 3B and 4A). To confirm that PKA inhibition was not the result of IL-17- or H89-mediated cytotoxicity, DNA content was measured. Neither IL-17 nor H89 altered DNA content (Figure 4B).

Role of PKA on Chondrogenic Differentiation

Finally, the effect of H89 on chondrogenic differentiation of human MSCs was evaluated. The addition of H89 resulted in considerable inhibition of proteoglycan expression and type II collagen deposition at day 21 (Figure 5A). Furthermore, the expression of chondrogenic markers (*COL2A1, ACAN, COL10A1,* and *ALP*) was suppressed by H89 in a dose-dependent manner and was completely abolished at concentrations above 3 μM (Figure 5B). We also confirmed that H89 did not cause growth suppression or cytotoxicity at concentrations less than 10 μM (data not shown). Taken together, these data suggest that suppression of PKA activity and SOX9 phosphorylation by IL-17 appears to result in the suppression of chondrogenesis.

Discussion

IL-17 is a cytokine that has attracted attention due to its involvement in chronic inflammation; induction of cartilage matrix breakdown and chondrocyte apoptosis in RA [19–21]. However, the effects of IL-17 on chondrogenic differentiation of human MSCs remain unclear. In the present study, we demonstrated that IL-17 inhibited chondrogenic differentiation of MSCs induced by pellet culture with chondrogenic induction medium containing TGF-β3. The effects of IL-17 were mediated through the suppression of SOX9 phosphorylation and inhibition of upstream PKA activity.

IL-1β and TNF-α inhibited chondrogenesis in our pellet culture system (Figure 1A and B) as reported previously [9], and IL-17 significantly suppressed cartilage matrix accumulation and chondrogenic marker expression in a dose-dependent manner, which reached maximum at 100 ng/mL (Figures 1 and 2). Although

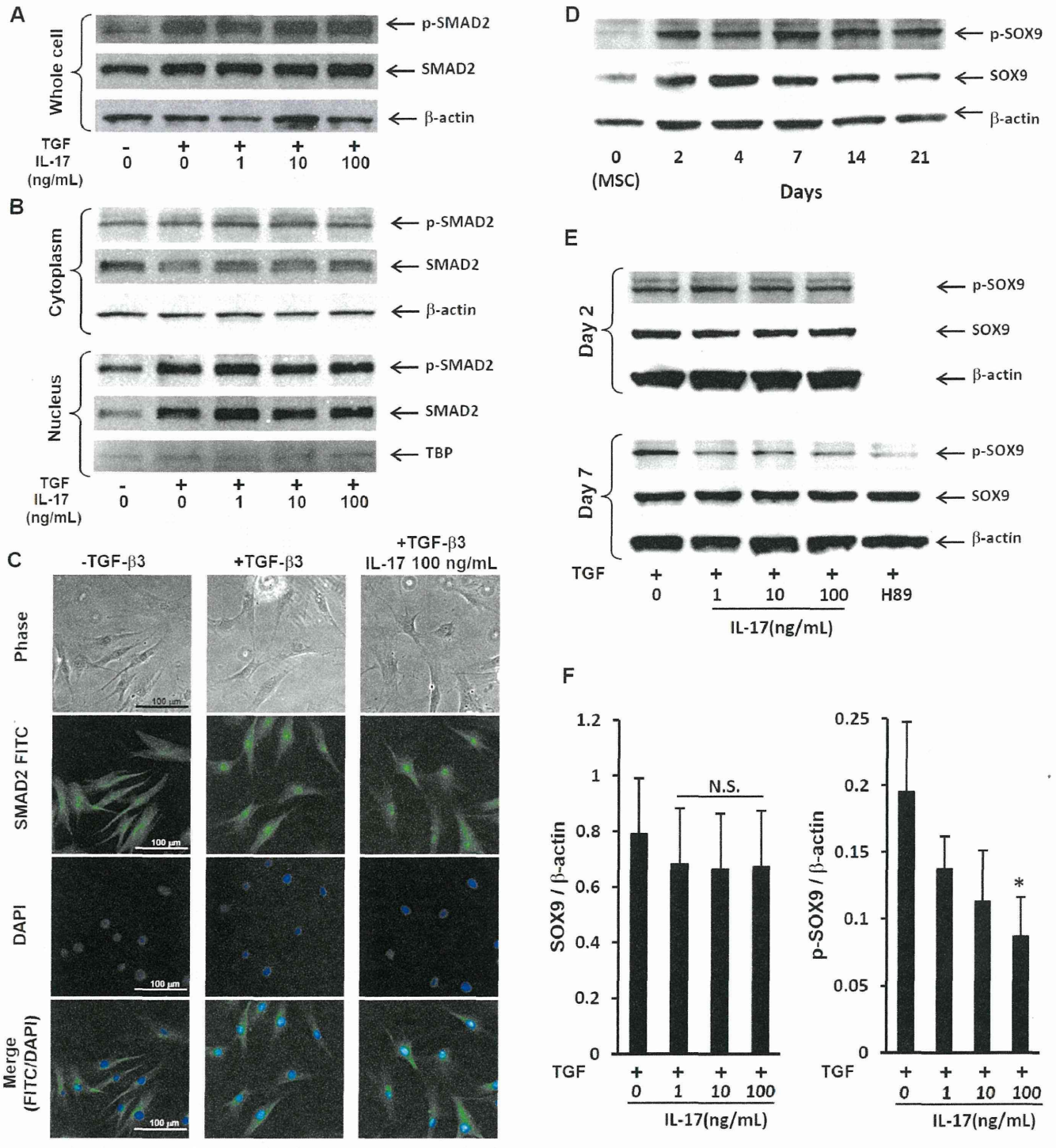

Figure 3. IL-17 does not affect SAMD2 activation, but attenuates SOX9 phosphorylation induced by chondrogenic culture. Human MSCs were cultured in monolayer (DMEM containing 5% FBS) with indicated concentrations of IL-17 throughout the culture period. After 16 hr culture with serum-starved medium (0% FBS), cells were stimulated with TGF-β3 (10 ng/mL) for 15 min and A, whole cell lysates or B, cytoplasmic (upper panel) and nuclear (lower panel) fraction were analyzed for SMAD2 and phospho-SMAD2 expression by western blotting. β-actin and TBP were used as loading controls. C, Human MSCs were cultured on cover-glass slides and SMAD2 localization was determined by immunofluorescence microscopy. Original magnification×20. Scale bars represent 100 μm. D, Human MSCs were cultured as aggregates in chondrogenic induction medium supplemented with TGF-β3 and aggregate lysates were evaluated at the indicated time points by western blot analysis for total and phosphorylated SOX9. β-actin was used as a loading control. (A, B, C and D) Data are representative of two independent experiments with similar findings. E, IL-17 or 10 μM H89 was added at the indicated concentrations and analysis carried out at day 2 and 7 by western blotting (top: day 2, bottom: day 7). F, Densitometric analysis on day 7 was performed with CS Analyzer, version 3.0 (bottom). Values represent the mean±SD of three independent experiments. *$P<0.05$, compared to no cytokine, by Dunnett's multiple comparison test. NS: not significant.
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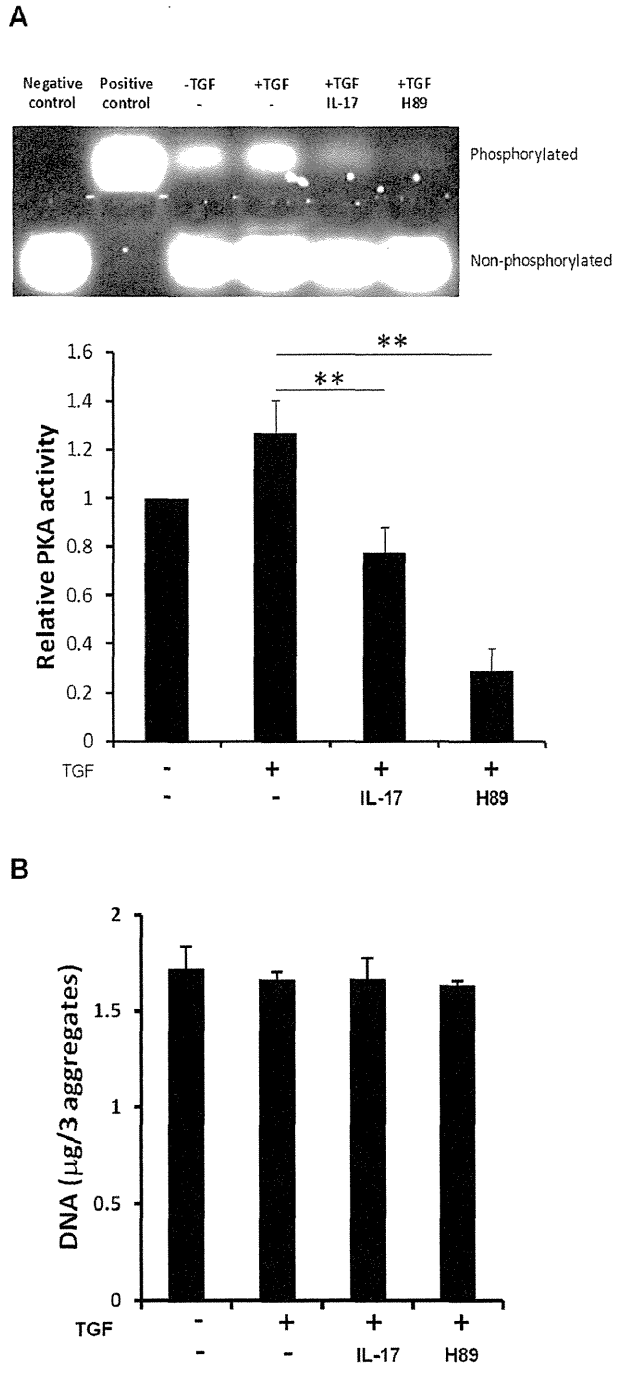

Figure 4. IL-17 treatment reduces PKA activity during chondrogenesis. A, Human MSCs were cultured as aggregates with TGF-β3 in the presence of IL-17 (100 ng/mL) or H89 (10 μM). After 7 days, 3 aggregates were pooled and lysed in each group, and PKA activity within the soluble protein fraction was determined using PepTag non-radioactive PKA assay (top). Recombinant PKA catalytic subunit (2 μg/mL) was used as a positive control and water was used as a negative control. Densitometric analyses of the band intensities were performed and results were expressed as the test band intensity relative to that of the "no TGF-β3" sample (bottom). B, The DNA content of three aggregates in each group was measured after 7-day culture. Values shown in A and B are mean±SD of three independent experiments. $^{**}P<0.01$ compared to no cytokine (+TGF-β3), by Dunnett's multiple comparison test.

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these *in vitro* concentrations are higher than the *in vivo* concentration of IL-17 in the synovial fluid of RA patients [15–17], MSCs can be exposed to much higher local concentrations of IL-17 *in vivo* than in the synovial fluid, since Th17 cells accumulate around MSCs at local inflammatory sites, such as synovial membranes [29,30].

IL-17 receptor mRNA was expressed in a low level in undifferentiated MSCs, whereas chondrogenic culture significantly increased its expression on day2 and 7 (Figure 1E). This suggests that MSCs becomes sensitive to IL-17 stimulation in the early phase of chondrogenic development. In addition, we confirmed that IL-17 receptor induction was retained in the presence of IL-17 (data not shown).

The increased expression of cartilage matrix and chondrogenic marker genes in our chondrogenic model was TGF-β3-dependent (Figures 1 and 2). IL-1β and TNF-α inhibit the expression of SOX9, the master transcription factor for chondrogenesis [31], by reducing TGF-β receptor type II expression, resulting in the suppression of SMAD2/3 activation and increased activity of SMAD7, a molecule with inhibitory effects [32–34]. However, IL-17 affected neither the TGF-β/SMAD signaling pathway (Figure 3A-C) nor total SOX9 expression (Figure 3E and F). Thus, our observations suggest that IL-17 inhibits chondrogenic differentiation through a mechanism different from that involving IL-1β or TNF-α. SOX9 is known to regulate the transcription of chondrocyte-specific genes, such as type II collagen and aggrecan [23]. SOX9 activity is regulated by several posttranslational modifications and among these, its modification by phosphorylation is the most widely studied [35]. Therefore, we measured phosphorylated SOX9 and found that IL-17 caused a significant decrease in phosphorylation. These results suggest that SOX9 phosphorylation is important for the chondrogenic differentiation of MSCs. PKA [26,27], a cyclic guanosine monophosphate (GMP)-dependent protein kinase II (cGKII) [36], and Rho kinase (ROCK) [37] have been reported to phosphorylate SOX9, enhance DNA-binding, and increase transcriptional activity. Our results indicated that IL-17 significantly suppressed PKA activity. In agreement with these results, H89, a selective PKA inhibitor, strongly suppressed SOX9 phosphorylation (Figure 3E) and chondrogenesis (Figure 5). These data implicate PKA in the phosphorylation of SOX9 and chondrogenic differentiation of human MSCs, which is consistent with previous reports indicating that PKA is a positive regulator of chondrogenic differentiation [38,39]. However, conflicting findings have been reported, demonstrating that H89 induced aggrecan, a chondrogenic marker [40]. This discrepancy is presumably related to different experimental conditions; the effect of H89 was evaluated without a chondrogenic induction factor such as TGF-β in monolayer cultures, whereas our experiments used pellet cultures with TGF-β3, which is commonly used for chondrogenic differentiation and can induce chondrogenesis more effectively than monolayer cultures [41].

IL-17 is known to induce cytokines, chemokines, and mediators of cartilage destruction in various cell types [42]. Presumably, IL-17-stimulated MSCs can affect various cell types via trophic effects, by secreting a variety of cytokines and chemokines. Because the suppression of SOX9 phosphorylation by IL-17 was stronger on day 7 than day 2 (Figure 3E), an indirect mechanism of action was considered. Although IL-1β and TNF-α are known as soluble factors that inhibit chondrogenesis [9,43], these cytokines were not detected in culture supernatants of IL-17-treated MSCs (data not shown). It is conceivable that some other factors that suppressed cAMP contributed to the inhibitory effect of IL-17. Prostaglandin E2 (PGE2) is a major positive regulator of

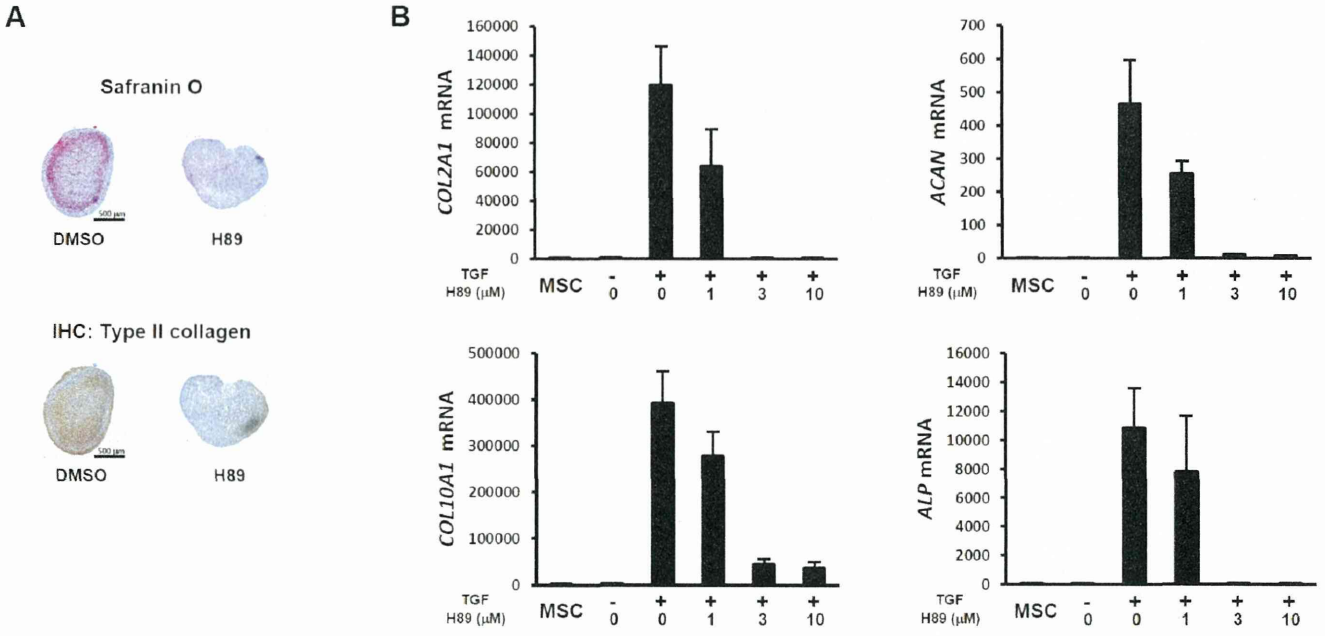

Figure 5. PKA activation is required for chondrogenic differentiation of human MSCs. A, Paraffin sections from aggregates cultured in the presence of 10 μM H89 for 21 days were stained with Safranin O and anti-type II collagen antibody. Original magnification×10. Scale bars represent 500 μm. B, *COL2A1, ACAN, COL10A1,* and *ALP* mRNA levels in aggregates treated with the indicated concentrations of H89 for 21 days were determined by real-time PCR. Values are mean±SD of three aggregates from 1 of 2 independent experiments with similar findings. See Figures 1 and 2 for the definition of other symbols.
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cAMP, which is secreted by MSCs and activates PKA through its EP2 and EP4 receptors [44]. However, details on the regulation of PGE2 production and EP2 and EP4 expression by IL-17 are not available at present. Further studies are needed to elucidate how and what molecules are regulated by IL-17 to mediate its inhibitory effect on chondrogenesis.

Repair of articular cartilage is considered difficult due to the limited regenerative capacity of cartilage. A variety of surgical procedures aimed at repairing defective cartilage in patients with RA, OA, or trauma are currently available [45]. However, the clinical improvement provided by these therapies is not permanent. For this reason, it is necessary to develop alternative approaches for complete and permanent cartilage repair and regeneration [46]. One of the most promising biological approaches for this purpose is cell-based therapy using MSCs [46,47]. In fact, implanted MSCs can differentiate into chondrocyte-like cells and improve cartilage structure in several animal studies [7,8]. MSCs are multipotent cells that can differentiate into various cells of mesenchymal lineages, including osteoblasts and chondrocytes, and can be easily isolated from bone marrow, adipose tissue, and other mesodermal tissues [4]. Moreover, several studies have described the immunosuppressive properties of MSCs [48]. These characteristics emphasize the potential usefulness of MSCs in joint regeneration treatments for RA patients. However, the efficacy of MSC-based cartilage repair therapy has not been determined in clinical trials and, therefore, it requires further studies [49]. Our results predict that IL-17 can inhibit MSC-based cartilage repair, therefore, preoperative inactivation of IL-17 in the joints of patients with RA is a promising approach in clinical settings.

In conclusion, the results of the present study demonstrated that IL-17, a key cytokine in chronic inflammation, inhibited chondrogenic differentiation of human MSCs through the suppression of PKA and SOX9 activity. These findings suggest that IL-17 could induce cartilage disorders by disrupting homeostasis and self-repair function.

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Author Contributions

Conceived and designed the experiments: MK KY KS SF KO YO YT. Performed the experiments: MK. Analyzed the data: MK. Contributed reagents/materials/analysis tools: MK KY KS SF KO YO YT. Wrote the paper: MK KY YT.

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ORIGINAL ARTICLE

Improvement of plasma endothelin-1 and nitric oxide in patients with systemic sclerosis by bosentan therapy

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Abstract The aim of this study was to evaluate the effects of bosentan on plasma endothelin-1 (ET-1) and nitric oxide (NO) as pulmonary hypertension (PH)-associated biochemical markers in patients with systemic sclerosis (SSc). Twenty-four SSc patients receiving bosentan for 24 weeks were registered in this prospective observational study. Ten patients were complicated with clinically suspected PH. Plasma levels of ET-1 and NO were assessed at baseline and after 24 weeks of treatment in SSc patients and in 15 healthy controls. Plasma levels of ET-1 and NO at baseline were significantly higher in SSc patients than in healthy controls ($p < 0.000$), and they were also significantly higher in SSc patients with PH than in those without PH ($p < 0.01$). Plasma ET-1 levels were significantly decreased after 24 weeks of bosentan therapy ($p < 0.0001$), and ET-1 levels of SSc patients with PH decreased to a level comparable to that in patients without PH. In the 10 SSc patients with PH, changes in plasma ET-1 levels during the 24 weeks of the study were significantly larger in the 5 patients whose functional class (FC) improved than in the 5 patients whose FC was unchanged ($p < 0.05$). Plasma NO levels were also slightly decreased in SSc patients after 24 weeks of bosentan therapy. Plasma ET-1 levels could reflect the presence and severity of PH in SSc patients. Additionally, changes in plasma ET-1 levels may indicate the response to bosentan therapy in SSc patients with PH.

Keywords Bosentan · Systemic sclerosis · Pulmonary hypertension · Endothelin-1 · Nitric oxide

Abbreviations

cGMP	Cyclic guanosine monophosphate
ELISA	Enzyme-linked immunosorbent assay
ET	Endothelin
FC	Functional class
NO	Nitric oxide
PAH	Pulmonary arterial hypertension
PAP	Pulmonary artery pressure
PH	Pulmonary hypertension
SSc	Systemic sclerosis
WHO	World Health Organization
6MWD	6-minute walk distance

Introduction

Pulmonary hypertension (PH) is a critical complication in patients with systemic sclerosis (SSc) [1]. The primary classifications of SSc-associated PH are pulmonary arterial hypertension (PAH), PH due to lung disease and/or hypoxia, and PH due to left heart disease. PAH affects approximately 10–15 % of patients with SSc [2] and is associated with a worse prognosis than Ssc with idiopathic PAH [3]. The three pathways to PAH treatment are the endothelin (ET) pathway, the nitric oxide (NO) pathway, and the prostacyclin pathway [4]. Treatment via the ET

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pathway uses an ET-receptor antagonist. Bosentan is a non-peptide antagonist that blocks both endothelin A (ET_A) and B (ET_B) receptors. The strength of recommendation of bosentan therapy is "A," which is a strong recommendation, in World Health Organization (WHO) functional class (FC) II and III patients in the PAH evidence-based treatment algorithm [5, 6]. Bosentan is also effective in patients with SSc-associated PAH [7–10].

Endothelin-1 has been shown to play a significant pathogenic role in PAH [11]. Endothelin-1 is a strong vasoconstrictor. Furthermore, it can stimulate the proliferation of pulmonary smooth muscle cells, fibroblast collagen production, and the contraction of fibroblast-populated collagen lattices [12]. In contrast, NO is a potent, endogenous, endothelium-derived vasodilator that directly relaxes vascular smooth muscle through the stimulation of soluble guanylate cyclase and increased production of intracellular cyclic guanosine monophosphate (cGMP) [13]. PAH is associated with a defect in the production of NO and therefore with decreased NO-induced vasodilatation [13].

In patients with SSc, ET-1 is a key pathogenic mediator that influences vasoconstriction, fibrosis, vascular hypertrophy, and inflammation [12]. In this study, we evaluated the effects of bosentan on plasma ET-1 and NO as PH-associated biochemical markers in SSc patients with or without PH.

Materials and methods

Patients and controls

Twenty-four SSc patients suspected by physicians of having PH were consecutively enrolled in the present study at the Center for Rheumatic Disease of Sasebo Chuo Hospital from June to December 2007. All patients provided their informed consent to participate in the present protocol, which was approved by the Institutional Review Board of Sasebo Chuo Hospital. All patients fulfilled the American College of Rheumatology preliminary classification for SSc [14]. The present study was a prospective observational study lasting 24 weeks. In this study, PH was defined as (1) a resting systolic pulmonary artery pressure (PAP) of >30 mmHg on echocardiogram, (2) mild to moderate dyspnea on exertion, (3) WHO FC II/III, and (4) symptoms not attributable to lung disease or hypoxia [15]. Since the present study was conducted before the establishment of guidelines for the diagnosis and treatment of PH [16], the diagnostic criteria of PH used here and mentioned above are different from those described in the guidelines. Systolic PAP was assessed by maximal tricuspid regurgitation jet velocity, which was measured as the peak regurgitate velocity in a continuous-wave Doppler flow profile obtained from the cardiac apex. No patient had undergone right-heart catheterization. In all patients, left ventricular function was normal on echocardiogram. Patients received a starting dose of bosentan of 62.5 mg twice daily, and the dose was increased to and then maintained at 125 mg twice daily after 4 weeks. Plasma samples were collected at baseline and at 24 weeks and were stored at −80 °C until assay. We also collected plasma samples from 15 healthy controls whose mean ± SD of age was 42 ± 10 years and whose sex ratio (men–women) was 7:8.

Clinical and laboratory assessment

Clinical response to the therapy was evaluated based on the WHO FC and 6-min walk distance (6MWD) in SSc patients with PAH. Exercise capacity was evaluated by 6MWD in accordance with the American Thoracic Society guidelines [17]. ET-1 and NO were assessed at baseline and after 24 weeks of treatment. Plasma levels of ET-1 were measured by enzyme-linked immunosorbent assay (ELISA; RIA2 method, BML, Tokyo, Japan). Plasma levels of NO were measured by chemiluminescence, using a highly sensitive NO measurement system (FES-450; Scholar-Tec Co., Ltd., Osaka, Japan) [18].

Statistical analyses

Within-group comparisons were made using the Mann–Whitney U test; changes from baseline were compared using the Wilcoxon's signed-rank test. Correlations were assessed with Spearman's correlation coefficient test. The overall significance level for statistical analysis was 5 % (two sided). p values of <0.05 were considered statistically significant.

Results

Baseline characteristics of the 24 SSc patients

The demographic data of the present 24 SSc patients at baseline are described in Table 1. In these patients, the mean ± SD of age was 60 ± 13 years and that of disease durations was 13 ± 6 years. Ten patients were complicated with PH; these patients were 62.4 ± 13 years old and all female. Five were WHO FC II, and the other 5 were FC III. The median (range) of 6MWD was 568 (600–440) m in these 10 patients.

Clinical efficacy of bosentan therapy in the 10 SSc-PH patients

The clinical efficacy of bosentan therapy was evaluated in the 10 SSc patients with PH. The WHO FC improved from

Table 1 Demographic and clinical characteristics at baseline of 24 SSc patients

	$N = 24$
Age (years[a])	60 ± 13
Gender: male/female (*N*)	4/20
Disease durations (years[a])	13 ± 6
Cutaneous type: limited/diffuse (*N*)	10/14
Complications (*N*)	
Interstitial lung disease	13
Digital ulcers	4
Pulmonary hypertension	10

[a] Mean ± SD

II to I in 3 patients and from III to II in 2 patients, though no statistically significant change was observed (Table 2). No patient showed an exacerbated FC. We observed significant increases in 6MWD at 12 and 24 weeks ($p < 0.01$, Table 2). The median changes (range) in 6MWD were 40 (28–70) m at 12 weeks and 60 (40–92) m at 24 weeks.

Plasma ET-1 and NO at baseline in the 24 SSc patients

Plasma levels of ET-1 and NO at baseline were significantly higher in the 24 SSc patients than in the 15 healthy controls ($p < 0.0001$). The median (range) of plasma ET-1 levels was 2.4 (1.8–4.5) pg/ml in the SSc patients and 0.4 (0.2–1.0) pg/ml in healthy controls. The median (range) of plasma NO levels was 60.9 (47.0–85.0) μM in SSc patients and 36.0 (29.5–45.6) μM in healthy controls. There was a positive correlation between plasma ET-1 and NO levels at baseline in SSc patients ($r = 0.59, p < 0.01$). Plasma ET-1 and NO levels at baseline were significantly higher in SSc patients with PH than in those without PH, and the values of plasma ET-1 and NO levels were relatively clearly dividable between SSc patients with and without PH (Fig. 1; Table 2).

In the 10 SSc patients with PH, plasma ET-1 and NO levels at baseline were significantly higher in FC III patients than in FC II patients ($p < 0.05$). The median (range) of plasma ET-1 levels was 3.4 (2.1–3.7) pg/ml in FC II patients and 4.0 (3.8–4.5) pg/ml in FC III patients. The median (range) of plasma NO levels was 77.3 (75.0–81.5) μM in FC II patients and 82.3 (77.6–85.0) μM in FC III patients.

Changes in plasma ET-1 and NO in 24 SSc patients treated with bosentan

Plasma ET-1 levels were significantly decreased after 24 weeks of bosentan therapy in SSc patients both with and without PH (Table 2). In the patients with PH, plasma ET-1 levels decreased to a level comparable to that in patients without PH (Table 2). Plasma NO levels were also significantly decreased after 24 weeks of bosentan therapy in patients both with and without PH, but changes in plasma NO levels during the 24 weeks of treatment were very slight (Table 2). Among the 10 SSc patients with PH, changes in plasma ET-1 levels during the 24 weeks of treatment (⊿ET-1) were significantly larger in the 5 patients whose FC improved than in the 5 patients whose FC remained unchanged; the median (range) of ⊿ET-1 was −1.6 (−2.2 to −1.1) versus −0.7 (−1.9 to −0.3) pg/ml, respectively ($p < 0.05$). However, changes in plasma NO levels during the 24 weeks of treatment (⊿NO) were not different between the improved patients and the unchanged patients; the median (range) of ⊿NO was −1.7 (−2.1 to −1.2) versus −1.7 (−1.9 to −1.2) μM, respectively.

Discussion

Endothelin-1 is a highly potent vasoconstrictor produced by endothelial cells that is a key pathogenic mediator of

Table 2 Change in clinical findings and plasma ET-1/NO concentrations during bosentan treatment

	SSc with PH ($N = 10$)			SSc without PH ($N = 14$)		
	Baseline	24 weeks	*p*	Baseline	24 weeks	*p*
WHO FC (*N*)	I, 0; II, 5; III, 5; IV, 0	I, 3; II, 4; III, 3; IV, 0	0.22	–	–	–
6MWD (m[a])	568 (600–440)	620 (532–650)	<0.01	–	–	–
Plasma ET-1 (pg/ml[a])	3.05 (2.1–4.5)[#]	2.05 (1.4–3.4)	<0.01	2.25 (1.8–3.1)	2.00 (1.7–2.4)	<0.01
Plasma NO (μM[a])	80.1 (75.0–85.0)[#]	78.5 (73.4–83.2)	<0.01	56.8 (47.0–61.4)	56.5 (44.1–62.9)	<0.05

Changes in WHO FC, 6MWD, and plasma concentrations of ET-1 and NO were examined during bosentan treatment as described in the text

The changes from baseline were compared using Wilcoxon's signed-rank test

ET endothelin, *FC* functional class, *NO* nitric oxide, *PH* pulmonary hypertension, *SSc* systemic sclerosis, *WHO* World Health Organization, *6MWD* 6-minute walk distance

[#] $p < 0.01$; The difference in baseline plasma ET-1 or NO concentrations between SSc patients with PH and those without SSc using Mann–Whitney's *U* test

[a] Median (range)