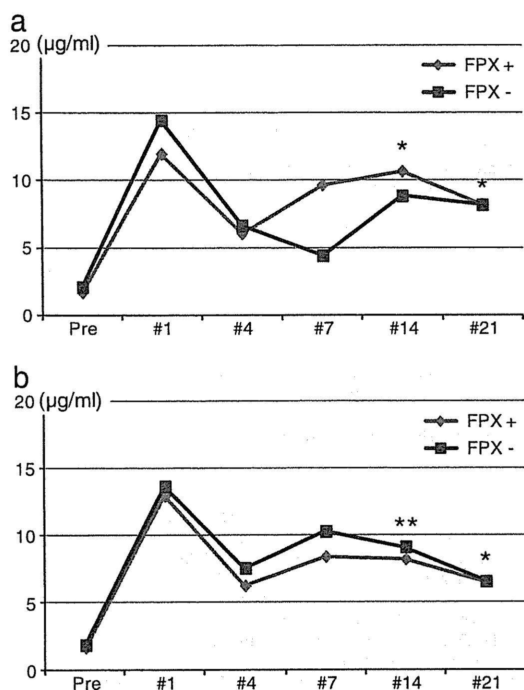

Fig. 2. a: Longitudinal changes in the D-dimer levels in the patients without DVT. The D-dimer levels were significantly lower in the patients treated with fondaparinux than not treated with fondaparinux on postoperative days 14 and 21.** = p<0.05 (Mann–Whitney *U* test) **b: Longitudinal changes in the D-dimer levels in the patients with DVT.** The D-dimer levels were significantly higher in the patients treated with fondaparinux than those not treated with fondaparinux on postoperative days 14 and 21.** = p<0.05 (Mann–Whitney *U* test)* = p<0.01 (Mann–Whitney *U* test).

fondaparinux prophylaxis. However, no such tendency was observed in DVT positive patients. The D-dimer levels were significantly lower in the patients treated without DVT than those not treated without DVT on postoperative days 14 and 21. Moreover, D-dimer levels were significantly higher in the fondaparinux-treated patients with DVT than in untreated patients with DVT on the postoperative days 14 and 21. The results implied the fondaparinux treatment in patients with DVT enhanced the blood fibrinolysis, but in the patients without DVT, blood fibrinolysis appears to be suppressed effectively in patients who receive fondaparinux. *Yamaguchi et al.* [4] *previously reported that in patients who received fondaparinux for 14 days after THA surgery, the incidence of DVT was 13.6% on day 1 after the surgery, 27.1% on day 4, and 11.9% on day 14 while in patients after TKA surgery, the incidence of DVT was 50.0% on day 1, 58.3% on day 4, and 20.8% on day 14. In the patients with DVT, DVT was resolved by fondaparinux treatment, which meaned enhanced fibrinolysis. Fondaparinux is assumed to render the clot more susceptible to fibrinolysis induced by tissue plasminogen activator (t-PA). This fibrinolytic activity of fondaparinux is mainly due to a modification of clot structure characterized by a loose fibrin conformation with less branched fibers and the presence of large pores* [23]*. Moreover, fondaparinux would have the ability of reducing thrombin generation and have been shown to promotoe fibrinolysis by reducing thrombin-activatable fibrinolysis inhibitor (TAFI) activation* [24]*. In contrast, fibrinolysis might not be enhanced in patients without DVT.* Our findings suggested there might be an impact on the diagnosis of DVT using these biomarkers after administration of fondaparinux. In particular, we observed that the D-dimer test on postoperative day 7 is useful for DVT screening in the fondaparinux-treated patients.

Table 3
Performance of SF and D-dimer test.

			Pre	#1	#4	#7	#14	#21
SF								
	FPX -	DVT (* p value)	n.s.	<0.01	n.s.	n.s.	n.s.	n.s.
		AUC	-	0.68	-	-	-	-
		BFV	-	1.43	-	-	-	-
	FPX +	DVT (* p value)	n.s.	<0.01	n.s.	n.s.	n.s.	n.s.
		AUC	-	0.64	-	-	-	-
		BFV	-	1.28	-	-	-	-
D-dimer								
	FPX -	DVT (* p value)	n.s.	<0.01	<0.01	<0.01	n.s.	n.s.
		AUC	-	0.65	0.74	0.67	-	-
		BFV	-	1.30	1.45	1.35	-	-
	FPX +	DVT (* p value)	n.s.	n.s.	n.s.	0.01	n.s.	n.s.
		AUC	-	-	-	0.55	-	-
		BFV	-	-	-	1.23	-	-

SF: Souble fibrin; FPX: Fondaparinux; DVT: Deep vein thrombosis; AUC: Area under curve;
BFV: Best fit value.
* Mann-Whitney *U* test.
The blood collection on postoperative day 1 was performed before the first injection of fondaparinux.

The SF value peaked on postoperative day 1, and decreased consistently thereafter. While the D-dimer value showed a bimodal peak. The D-dimer value rapidly increased on postoperative day 1, which was similar to the SF value, but the D-dimer level decreased on postoperative day 4, peaked again on postoperative days 7–14, and then decreased. Our results confirmed the findings from studies that reported persistently elevated D-dimer levels 7 days after major othopedic surgery [3,9,13,25,26]. To the best of our knowledge, no previous reports have referred to the bimodal peak of the D-dimer level following surgery. Since surgery is associated with haemostasis and surgical wound bleeding, both of which lead to fibrin formation and an increase in both SF and D-dimer without any association with DVT [9,27]. However, the patients who developed DVT showed a more marked increase. The longitudinal difference between SF levels and D-dimer levels might be due to the differences in the coagulation and fibrinolytic systems. Our findings implied that the activation of the coagulation system induced by surgery subsides within a few days, while the activation of the fibrinolytic system induced by surgery is induced in a more complicated manner after surgery.

This study has three main limitations. First, the number of enrolled patients was small. Second, both the sensitivity and the specificity for DVT were lower than expected. In Japan, SF and D-dimer concentrations are usually measured using LIA, while in Europe and North America, the D-dimer level is measured using ELISA. LIA has not been recommended instead of the ELISA method, which may explain our lower sensitivity and specificity. Third, the patients enrolled in this study did not receive the same treatment for DVT. Between September 2003 and November 2007, patients with DVT were treated with UFH or low-dose warfarin, while from December 2007, fondaparinux treatment was scheduled to continue for 14 days. This may have affected the SF and D-dimer levels.

In conclusion, our study demonstrated that fondaparinux effectively prevents DVT in patients undergoing THA or TKA. The SF and D-dimer tests might be affected after administration of fondaparinux, complicating the diagnosis of DVT. However, we observed that the D-dimer test on postoperative day 7 is useful for DVT screening in the patients treated with fondaparinux. The SF on postoperative day 4, 7, and 14 and D-dimer levels on postoperative day 14 and 21 in patients treated with fondaparinux without DVT were lower than in patients without fondaparinux treatment, while the D-dimer levels on postoperative day 14 and 21 in patients treated with fondaparinux with DVT were higher than in patients without fondaparinux treatment. Nevertheless, to confirm our observations and to ensure a more accurate diagnosis, further investigations are required.

Conflict of interest statement

The authors state that they have no conflict of interest.

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SHORT COMMUNICATION

Repeat etanercept administration restores clinical response of patients with rheumatoid arthritis

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Abstract We determined whether repeated treatment with the tumor necrosis factor-α (TNF-α) antagonist etanercept can be effective after an initial clinical response to this drug is lost. We describe three female patients with active, refractory rheumatoid arthritis who were administered with a second course of etanercept after eventually becoming refractory to a first course. Disease activity was high in all three patients before initial etanercept therapy, and each of them had clinically responded by 24 weeks. However, the initial clinical effect was lost between 1.5 and 3.5 years thereafter, and tocilizumab was administered, but the effect was lost again between 3 and 18 months later. Two patients did not respond to subsequent treatment with adalimumab and infliximab. Etanercept administered once again reduced disease activity in all three patients, none of whom developed any acute side effects. Etanercept re-administration significantly improved clinical disease activity and inflammatory parameters in three patients with RA who were refractory to biological anti-TNF agents.

Keywords Rheumatoid arthritis · Biological agent · Refractory disease · Etanercept · Re-administration

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Introduction

Tumor necrosis factor-α (TNF-α) antagonists are remarkably effective against rheumatoid arthritis (RA) [1–6]. These drugs changed the treatment goal for RA to include not only the inhibition of bone destruction, but also the induction of remission [7]. However, responses are insufficient in 20–40% of patients with RA. Switching even to similar biological agents has become a popular approach to treating RA and although this strategy can be effective, options are limited for refractory patients [8–10].

Herein, we describe three patients with RA in whom the re-administration of etanercept restored a lost initial clinical response.

Patients and methods

This retrospective, observational study analyzed data from all patients with RA who were treated with etanercept at our institution between 2005 and 2009. All patients fulfilled the 1987 American College of Rheumatology (ACR) criteria for RA. Exclusion criteria consisted of compliance with the contraindications and precautions provided by the manufacturers of biological agents. All patients provided written informed consent to their records being analyzed, and the local ethics committee approved the study.

Results

Etanercept was administered for the first time to 135 patients with RA between 2005 and 2009. Six and 31 of them discontinued therapy because of adverse events and an absent or secondary loss of efficacy, respectively, and

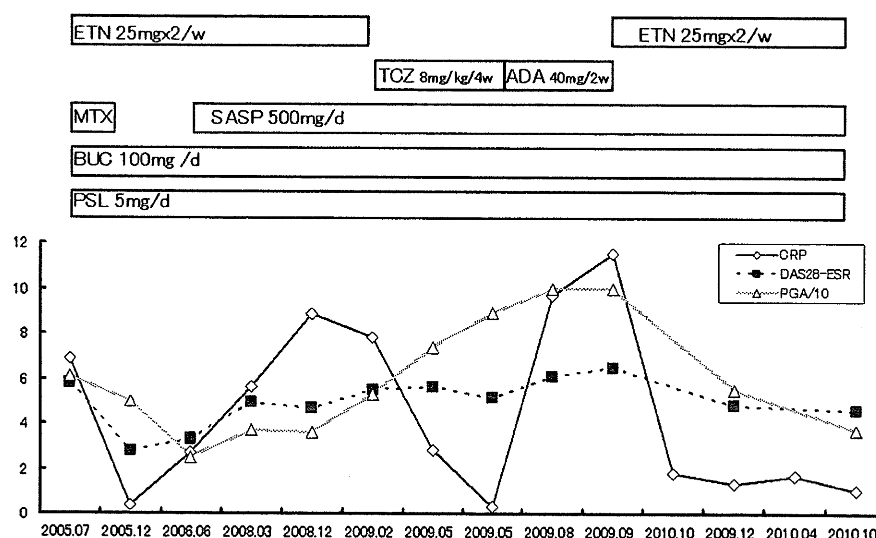

Fig. 1 Change in DAS28-ESR, PGA and CRP values in a 63-year-old woman with RA. *DAS28*, disease activity score in 28 joints; *PGA*, patient global assessment; *CRP*, C-reactive protein; *ETN*, etanercept; *TCZ*, tocilizumab; *ADA*, adalimumab; *IFX*, infliximab; *MTX*, methotrexate; *SASP*, sulfasalazine; *BUC*, bucillamine; and *PSL*, prednisolone

almost all of them switched to another biological agent. Three of these patients switched to different biological agents and returned to etanercept because their initial response to this agent was better than that to any of the others.

Case 1

A 63-year-old woman was diagnosed with seropositive RA in 1997 and had been receiving oral prednisolone (5 mg/day), bucillamine (100 mg/day) and methotrexate (6 mg/week). Bucillamine is a disease-modifying antirheumatic drug (DMARD) with SH groups that was developed and released in Japan during 1987 and in Korea during 1992.

In August 2004, the patient was administered with infliximab (3 mg/kg/8 weeks) together with the preceding DMARD therapy due to high disease activity. An infusion reaction necessitated discontinuation in May 2005.

Etanercept (25 mg twice weekly) administered subcutaneously was added to the DMARD therapy (Fig. 1). Improvement was remarkable at 24 weeks of etanercept therapy, as evidenced by the following changes: disease activity score (DAS) 28-ESR from 6.10 to 2.17; patient global assessment (PGA) from 61 to 5 mm; and C-reactive protein (CRP) from 6.9 to 0.4 mg/dL. This patient initially responded to etanercept despite discontinuing methotrexate at 4 weeks due to a liver disorder. However, etanercept lost its effect after 3.5 years despite adding sulfasalazine.

In February 2009, she received tocilizumab (8 mg/kg/4 weeks) along with prednisolone, bucillamine and sulfasalazine. However, she did not respond to tocilizumab, which was stopped at 12 weeks. Adalimumab introduced in May 2009 (40 mg/2 weeks) together with the preceding DMARD therapy had no effect and was stopped after 12 weeks.

Laboratory tests revealed the following: DAS28-ESR, 6.48; PGA, 100 mm; and CRP, 11.54 mg/dL. In September 2009, etanercept (25 mg twice weekly) was started again, because she had initially responded to this agent more effectively than to other biological agents. She again improved remarkably at 12 and 48 weeks of etanercept as follows: DAS28-ESR, 4.82 and 4.61; PGA, 55 and 37 mm; and CRP, 1.32 and 1.04 mg/dL, respectively.

Case 2

A 59-year-old woman was diagnosed with seropositive RA in 2001. Because of high disease activity (DAS28-ESR, 5.52; PGA, 98 mm; and CRP, 2.3 mg/mL), she was treated with etanercept (25 mg twice weekly) starting from May 2006 (Fig. 2). Preceding antirheumatic therapy with prednisolone (5 mg/day), sulfasalazine (500 mg/day) and methotrexate (4 mg/week) was continued. Her symptoms and disease activity values improved at 24 weeks as evidenced by the following: DAS28-ESR, 3.58; PGA, 29 mm; and CRP, 0.3 mg/dl. However, arthritis gradually recurred despite increasing the methotrexate dose from April 2007.

At the end of 2008, RA activity increased and tocilizumab administered together with methotrexate and prednisolone from December of that year, resulted in a measurable decrease in disease activity after 24 weeks (DAS28-ESR, 5.95 to 2.07; PGA, 93 to 24; and CRP, 2.76 to 0.02 mg/dL). Her symptoms improved once, but arthritis gradually recurred. She discontinued tocilizumab in February 2010.

Infliximab was introduced in March 2010 with the preceding DMARD therapy, but stopped after three courses of treatment due to the lack of a response.

She had initially responded to etanercept therapy but not to anti-TNF antibody, and since disease activity was

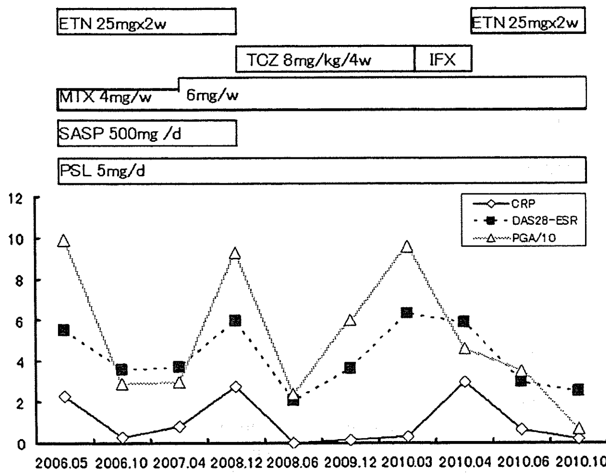

Fig. 2 Change in DAS28-ESR, PGA and CRP values in a 59-year-old woman with RA

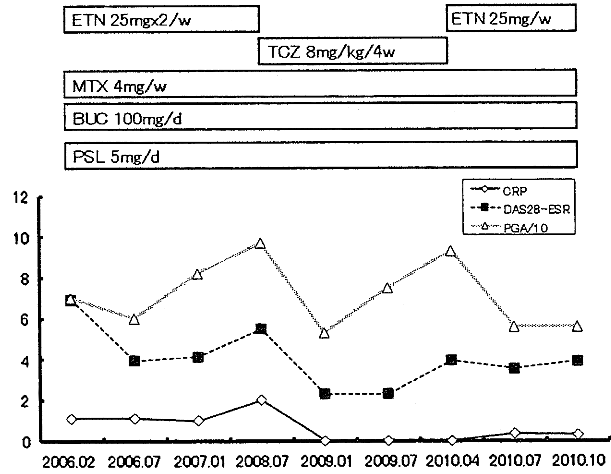

Fig. 3 Change in DAS28-ESR, PGA and CRP values in a 64-year-old woman with RA

increasing (DAS28-ESR, 5.86; PGA, 46 mm; and CRP, 2.96 mg/mL), etanercept (25 mg twice weekly) was administered again from April 2010. The disease activity improved, and parameters of general clinical activity decreased. Laboratory tests revealed the following at 24 weeks: DAS28-ESR, 2.55; PGA, 7 mm; and CRP, 0.2 mg/dL.

Case 3

A 64-year-old woman was diagnosed with seropositive RA in 2001. Because treatment with oral prednisolone (5 mg/day), bucillamine (100 mg/day) and methotrexate (4 mg/week) failed, we administered etanercept (25 mg twice weekly) from February 2006 (Fig. 3). The DAS28-ESR values improved from 6.93 to 3.84 at 24 weeks later. However, her clinical findings gradually deteriorated, and the DAS28-ESR value increased to 5.48 at 1.5 years of etanercept therapy. Therefore, treatment was switched in July 2008 from etanercept to tocilizumab (8 mg/kg/4 weeks) combined with the preceding DMARD therapy. Her symptoms improved once, but arthritis gradually recurred. Tocilizumab was discontinued in April 2010.

Etanercept (25 mg/week) was started again in April 2010 because attending a hospital twice each week was difficult for this patient. Improvements were remarkable at 24 weeks of etanercept re-administration (PGA, 93 to 56 mm). The patient was satisfied with the treatment outcome although the DAS28-ESR and CRP values did not change.

None of the patients developed remarkable adverse events.

Discussion

Biological agents that target TNF-α and interleukin-6 (IL-6) represent a significant advance in the management of rheumatoid arthritis. Therapy with these agents is associated with remarkable improvements in the clinical signs and symptoms of RA, and they can also retard the destructive processes that often characterize this condition. Although effective and well-tolerated in a substantial proportion of patients with RA, about 20–40% of them respond poorly or not at all to biological agents, or experience adverse events over time. Thus, switching from one biological agent to another or several others has emerged as a strategy to address treatment failures within this drug class.

Etanercept is a human TNF soluble receptor that was approved in Japan during 2005 and is now widely used to treat RA. Etanercept like other TNF inhibitors such as adalimumab and infliximab not only binds with high affinity to soluble and cell-associated TNF, but it also binds to lymphotoxin-alpha (previously known as TNF-β) [8].

Although the immunogenicity of biological agents has raised potential safety and efficacy concerns, etanercept does not appear to be highly immunogenic [11]. Non-neutralizing antibodies to etanercept arise in <5% of adult patients with RA or psoriatic arthritis, and antibodies to etanercept do not correlate with clinical responses or adverse events. No remarkable adverse events developed in our three patients.

The efficacy and safety of etanercept for treating psoriasis and psoriatic arthritis have been proven in several clinical trials. Recent studies have found that the discontinuation and re-administration of etanercept can improve joint pain and nail involvement of psoriasis and psoriatic

arthritis, similarly to continuous treatment without remarkable adverse events [12–14]. The present study is the first to describe the effects of etanercept re-administration in patients with RA that becomes refractory to initial treatment with this agent.

Patients with RA for whom a first TNF inhibitor has failed should receive another TNF inhibitor, such as abatacept, rituximab or tocilizumab according to the EULAR recommendations [15]. Recent studies have shown that if a first anti-TNF-α agent is discontinued due to refractoriness, then the likelihood of recurrent ineffectiveness of a second anti-TNF-α agent is increased threefold [16]. Two (Cases 1 and 2) of our patients did not respond well to anti-TNF-α agents administered after initial etanercept treatment had failed.

These findings indicate that etanercept re-administration to patients who had initially responded and then failed to respond to a different class of anti-TNF such as tocilizumab is useful. One limitation of this study is the short follow-up of the second, compared with the first etanercept administration. Another is that the mechanism of the response to etanercept is unknown. Thus, disease activity of RA might increase again and require the administration of abatacept, which is a different type of anti-TNF drug that has recently received approval for use in Japan. Further studies are required to determine the long-term safety and therapeutic effect of repeated therapy.

In conclusion, our results suggest that repeated administration of the soluble TNF receptor etanercept is a safe and effective treatment option for some patients, even after the initial response to this drug is lost.

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Conflict of interest The authors have declared no conflicts of interest.

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ORIGINAL ARTICLE

The efficacy of rebamipide add-on therapy in arthritic patients with COX-2 selective inhibitor-related gastrointestinal events: a prospective, randomized, open-label blinded-endpoint pilot study by the GLORIA study group

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Abstract
Objective We aimed to confirm the effect of combined treatment with celecoxib and rebamipide would be more effective than celecoxib alone for prevention of upper gastrointestinal (GI) events.
Methods Patients with rheumatoid arthritis, osteoarthritis, and low back pain were enrolled in this study. Patients were randomized to two groups: a monotherapy group (100 mg celecoxib twice daily) and a combination therapy group (add on100 mg of rebamipide three times a day). The GI mucosal injury was evaluated by endoscopic examination before treatment and at 3 months. The primary endpoint was to evaluate the preventive effect of the combination therapy group for GI events, endoscopic upper GI ulcers and intolerable GI symptoms, compared with the monotherapy group.
Results Seventy-five patients were enrolled. Sixty-five patients were analyzed (16 males, 49 females; mean age: 67 ± 13 years). The prevalence of upper GI events, five of endoscopic GI ulcers and one of intolerable GI symptoms, were 6/34 (17.6 %) in the monotherapy group and 0/31 in the combination therapy group, $p = 0.0252$.
Conclusions The combination therapy group was more effective than the monotherapy group for prevention of upper GI events in this study. Rebamipide might be a candidate for an option to prevent COX-2 selective inhibitor-induced upper GI events.

Keywords Gastrointestinal tract · NSAIDs · COX-2 inhibitors · Rheumatoid arthritis · Osteoarthritis

Introduction

Guidelines and consensus meetings about patients with osteoarthritis (OA) and rheumatoid arthritis (RA) have recommended the use of nonselective nonsteroidal anti-inflammatory drugs (NSAIDs) plus a proton pump inhibitor (PPI) or a cyclooxygenase-2 (COX-2)-selective inhibitor alone. Recent reports recommended the use of a COX-2-

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selective inhibitor plus a PPI in patients with one or more risk factors for adverse gastrointestinal (GI) events [1, 2]. A COX-2-selective inhibitor was developed to prevent nonselective NSAID-derived GI complications. The Vioxx Gastrointestinal Outcomes Research (VIGOR) trial and the Celecoxib Long-term Arthritis Safety Study (CLASS) found that the use of a COX-2-selective inhibitor avoided symptomatic GI events significantly better than nonselective NSAIDs [3, 4]. These studies showed that the prevalence of symptomatic GI events was only 2 % in patients taking a COX-2-selective inhibitor. The development of COX-2-selective inhibitors might resolve the risk of adverse GI events caused by nonselective NSAIDs. However, those previous studies evaluated only symptomatic GI events, and ignored asymptomatic GI events. Moreover, some patients taking a COX-2-selective inhibitor have risk factors for GI events, such as a history of ulcers, advanced age, and the concomitant use of NSAIDs, corticosteroids, or antiplatelet drugs. Therefore, an endoscopic evaluation is required to ensure the inclusion of asymptomatic GI events, including bleeding from a gastroduodenal ulcer. Goldstein et al. [5], Simon et al. [6] and Cheung et al. [7] reported that gastroduodenal ulcers were identified by endoscopic evaluation in almost 3–10 % of patients with RA or OA taking a COX-2-selective inhibitor [5–7]. However, there is no report to identify the prevalence ratio of COX-2-selective inhibitor induced GI injury in Japan. Therefore, it is necessary to identify it and a therapeutic strategy for preventing gastroduodenal ulcers.

Rebamipide, which induces endogenous prostaglandin (PG), is indicated for gastric ulcer and gastritis. Rebamipide prevented gastroduodenal ulcers as efficiently as misoprostol (a PG analogue) with incidences of gastroduodenal ulcer of 4.3 and 4.4 % in patients with RA or OA who were taking nonselective NSAIDs [8].

We aimed to confirm the hypothesis that combined treatment with celecoxib and rebamipide would be more effective than celecoxib alone for prevention of upper GI injury.

Patients and methods

Study setting

The study was approved by the Institutional Ethics Committee of Mie University Hospital, and was conducted from August 2008 to July 2011 at Mie University Hospital and 14 affiliated institutions. Written informed consent was obtained from all participants. This study was conducted with adherence to the Declaration of Helsinki. This study was registered with the University Hospital Medical Network Clinical Trials Registry (UMIN-CTR, UMIN000002112).

Study design

This study was a prospective randomized open-label blinded-endpoint study. Patients with RA, OA, or low back pain (LBP) were randomly assigned to either the monotherapy group or the combination therapy group (Fig. 1). The study drug in the monotherapy group was 100 mg b.i.d. celecoxib (Pfizer Co., Ltd., Tokyo, Japan). In the combination therapy group, the study drugs were 100 mg b.i.d. celecoxib plus 100 mg t.i.d. rebamipide (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). The study drugs were administered for 3 months. Endoscopic evaluations were conducted before the study and after 3 months. The endoscopic results were assessed by an endoscopic evaluation committee (consisting of the GLORIA study group).

Inclusion and exclusion criteria

Patients with RA, OA, or LBP were included. The exclusion criteria were: serious complications of cardiovascular disease (CV), history of CV, high risk of CV, adenocarcinoma, history of gastrointestinal surgery, GI ulceration, GI bleeding, gastroesophageal reflux disease, inability to stop treatment with PPIs, histamine 2 receptor antagonists, or gastroprotective agents, taking less than 75 % of study drug, opposition of the personal physician, and inability to complete the study for reasons other than GI events.

Outcomes

The primary endpoint was to evaluate the preventive effect of rebamipide for aggravation of GI events. GI events were defined as prevalence of endoscopic upper GI ulcers (≥5 mm in diameter) and intolerable GI symptoms. The

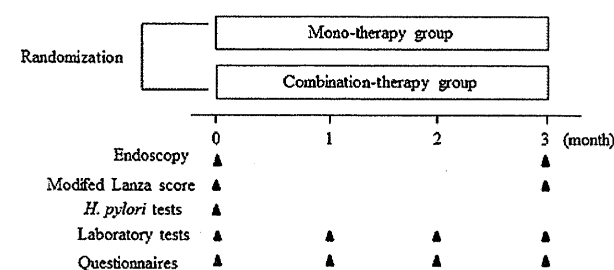

Fig. 1 Study design. Patients with rheumatoid arthritis, osteoarthritis, or low back pain were randomly assigned to either the monotherapy group or the combination therapy group. The study drug in the monotherapy group was 100 mg b.i.d. celecoxib. In the combination therapy group, the study drugs were 100 mg b.i.d. celecoxib plus 100 mg t.i.d. rebamipide. Endoscopic evaluation with a modified Lanza score were conducted before the study and after 3 months. Clinical laboratory tests, a questionnaire survey of gastrointestinal (GI) symptoms, and a visual analogue scale (VAS) assessment of joint pain were conducted before the start of the study and every month as safety assessments

secondary endpoints were to evaluate the prevalence of mucosal injury (erosion) compared with celecoxib monotherapy at 3 months and the evaluation for the efficacy of a COX-2-selective inhibitor, given as a treatment for joint pain using a VAS assessment. *Helicobacter pylori* (*H. pylori*) infection was determined by the presence of urinary *H. pylori* IgG antibodies or a urea breath test.

Safety assessment

Clinical laboratory tests, a questionnaire survey of GI symptoms, and a visual analogue scale (VAS) assessment of joint pain were conducted before the start of the study and every month. A VAS assessment was conducted to confirm joint pain with the VAS scores ranging from 0 mm (no pain) to 100 mm (intolerable pain). Tolerable joint pain was defined as <25 mm of the VAS score in this study. The GI symptoms were recorded as the frequency of stomach ache, heart burn, gastric hyperacidity, nausea or vomiting, gastric pain after a meal, abdominal distention, early satiety, belching, gastric discomfort in the morning, and tarry stool. Clinical laboratory tests were performed, and aspartate aminotransferase, alanine aminotransferase, C-reactive protein, creatinine, blood urea nitrogen, and hemoglobin were measured. The GI symptoms were checked every month.

Randomization

A computer-generated list of random numbers was used to allocate the participants to a treatment group. Randomization was performed according to the method of minimization for assignment. Randomization and assignment were performed by the Mie Clinical Trials Support Network Center.

Statistical analysis

The endpoint was to compare the prevalence of COX-2-selective inhibitor related GI ulcer and erosion in the combination therapy group with the monotherapy group. Pearson's Chi square or Fisher's exact test was applied for proportions. The contribution of the patients' backgrounds was assessed with a t test or Wilcoxon's rank sum test for continuous variables, and the Chi square test or Fischer's exact test was used for categorical variables. The prevention of joint pain in the combination therapy group and the monotherapy group were evaluated by using the Cochrane–Armitage method and two-way repeated-measures ANOVA. A $p < 0.05$ was considered to be statistically significant. Analysis was performed by JMP version 9 (SAS Institute Inc, Cary, NC, USA).

Results

Seventy-five patients were enrolled. Three patients with gastric ulcer and gastroesophageal reflux disease (GERD) were excluded. Three patients in the monotherapy group dropped out for their own reasons, and four patients in the combination therapy group dropped out. Sixty-five patients were analyzed (16 males, 49 females; mean age: 67 ± 13 years) and 34 patients were assigned to the monotherapy group, and 31 were in the combination group (Fig. 2).

The patients' backgrounds are shown in Table 1. The proportions with arthritis were: 29.5 % RA, 47.0 % OA, and 23.5 % LBP in the monotherapy group; and 45.2 % RA, 29.0 % OA, and 25.8 % LBP in the combination therapy group. The concomitant use of drugs was: 5.8 % aspirin, 23.5 % steroids, and 0 % anti-tumor necrosis factor α (anti-TNFα) in the monotherapy group, and 6.5 %

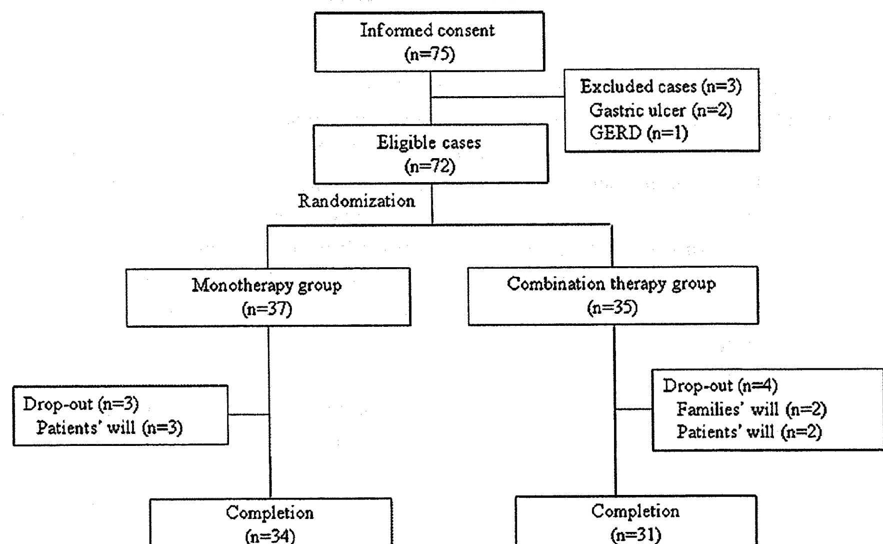

Fig. 2 The study flow diagram of 75 patients. Seventy-five patients were enrolled. Three patients with gastric ulcer and gastroesophageal reflux disease were excluded. Seven patients dropped out because of families' and patients' will. Sixty-four patients were analyzed and 34 patients were assigned to the monotherapy group, and 31 were in the combination group

Table 1 Patients' characteristics

Characteristics	Monotherapy group ($n = 34$)	Combination therapy group ($n = 31$)	p value
Age, mean ± SD (median)	67 ± 11(69)	68 ± 9 (69)	0.7824
Sex, male/female	10 (29.4)/24 (70.6)	6 (19.4)/25 (80.6)	0.3472
Diagnosis, *n* (%)			0.2874
Rheumatoid arthritis	10 (29.5)	14 (45.2)	
Larsen grade			0.4979
I	2 (20.0)	2 (14.3)	
II	3 (30.0)	3 (21.4)	
III	3 (30.0)	2 (14.3)	
IV	2 (20.0)	7 (50.0)	
V	0 (0.0)	0 (0.0)	
Das28-CRP[a]			0.1167
High	2 (20.0)	1 (7.1)	
Moderate	2 (20.0)	8 (57.1)	
Low	2 (20.0)	4 (28.6)	
Remission	4 (40.0)	1 (7.1)	
Osteoarthiritis	16 (47.0)	9 (29.0)	
Kallegren and Lawrence			0.5060
1	1 (6.2)	2 (22.2)	
2	6 (37.5)	3 (33.3)	
3	7 (43.8)	2 (22.2)	
4	2 (12.5)	2 (22.2)	
Low back pain	8 (23.5)	2 (6.5)	
Concomitant use of aspirin, *n* (%)	2 (5.8)	8 (25.8)	0.9240
Steroid, *n* (%)	8 (23.5)	8 (25.8)	0.8314
Anti-TNFα, *n* (%)	0 (0.0)	3 (9.7)	0.0632
History of ulcer, *n* (%)	7 (20.6)	8 (25.8)	0.6180
Erosion, n (%)	20 (58.8)	15 (48.4)	0.3992
H. pylori infection, *n* (%)	8 (23.5)	16 (51.6)	0.0191
NSAIDs-use, *n* (%)			
COX-2 selective inhibitor user	12 (35.3)	14 (45.2)	0.4173
COX-2 non-selective inhibitor user	11 (32.4)	12 (38.7)	0.5924
NO-users	11 (32.4)	5 (16.1)	0.1294

[] total number of patients for analysis

[a] DAS28-CRP was classified into 4 criteria, high was higher than 4.1, moderate was 2.7–4.1, low was 2.3–2.7, remission was lower than 2.3 [9]

aspirin, 25.8 % steroids, and 9.7 % anti-TNFα in the combination therapy group. In the monotherapy group, 20.6 % of patients had a history of ulcers, as did 25.8 % in the combination therapy group. The prevalence of patients with erosion was 58.8 % in the monotherapy group and 48.4 % in the combination therapy group. No differences were found between the groups except *H. pylori* infection. The prevalence of *H. pylori* infection was significantly high in the combination-therapy group.

The incidence of GI events and erosions in the two groups are shown in Table 2. The combination therapy was more effective for preventing upper-GI events than the monotherapy: GI events occurred in 6 of 34 (17.6 %) patients in the monotherapy group, involved 5 of GI ulcer and a case of drug cessation; and in 0 of 31 (0 %) patients in the combination therapy group, $p = 0.0252$. The diagnoses of 6 patients with GI events were RA in 2 patients, OA in 2 patients, and LBP in 2 patients. According to the disease activity score (DAS) 28-C-reactive protein (CRP), RA patients with GI events were high in one patient and remission in the other patient [9]. The incidence of patients with erosion at 3 months was 24 of 34 (70.6 %) patients in the monotherapy group and 9 of 31 (29.0 %) in the combination therapy group, OR: 0.17, 95 % CI: 0.05–0.49, $p = 0.0012$. The increase in the proportion of patients with erosion was 11.8 % in the monotherapy group and −19.4 % in the combination therapy group relative to the baseline endoscopic evaluations performed before the study. The ratio of *H. pylori*-infected patients was 20.0 % (1/5) in patients with upper GI ulcers and the ratio of

Table 2 The prevalence of upper GI events

	Monotherapy group [$n = 34$]	Combination therapy group [$n = 31$]	Odds ratio (95 % CI)	p value
Upper GI events	6 (17.6)	0 (0.0)		0.0252
Upper GI ulcer[a]	5 (14.7)	0 (0.0)		0.0543
Gastric ulcer	4 (11.8)	0 (0.0)		0.1150
Duodenal ulcer	2 (5.8)	0 (0.0)		0.4933
Stop medication[b]	1 (2.9)	0 (0.0)		1.0000
Erosion	24 (70.6)	9 (29.0)	0.17 (0.05–0.49)	0.0012

[] total number of patients for analysis, *GI* gastrointestinal, *n* number of patients, *CI* confidence interval

[a] Upper GI ulcer was defined as gastroduodenal ulcer. An ulcer was defined as an excavated and deep mucosal lesion measuring 5 mm or more in diameter

[b] Stop medication: one patient had intolerable abdominal pain

Table 3 Number of patients with joint pain evaluated by VAS score (<25 mm)

	Pre	1 M	2 M	3 M	p value
During movement [n (%)]					
Monotherapy group	9 (26.5)	14 (41.2)	17 (50.0)	18 (52.9)	0.0200
Combination therapy group	8 (25.8)	13 (41.9)	13 (41.9)	16 (51.6)	0.0494

H. pylori non-infected patients was 25.1 % (7/29) in the mono-therapy group. There was no relationship between *H. pylori* infection and incidence of upper GI ulcers.

The effect of the COX-2-selective inhibitor, celecoxib, assessed with a VAS evaluation, is shown in Table 3. There was no statistically significant difference between the monotherapy group and the combination therapy group. The number of patients who scored less than 25 mm on the VAS increased significantly from baseline in both groups. In the evaluation of GI symptoms, 52.9 % (18/34) of patients in the monotherapy group and 51.6 % (16/31) in the combination therapy group were asymptomatic. There was no statistically significant difference between the groups throughout the study period.

There were no significant changes in the results of the clinical laboratory tests during the study period. Medication was stopped in one patient in the monotherapy group because of GI symptoms (abdominal pain) 1 week after the COX-2-selective inhibitor was first administered.

Discussion

In the present study, upper-GI ulcers $\geq$ 5 mm in diameter occurred in 5 of 34 (14.7 %) patients, intolerable GI symptoms occurred in 1 of 34 (2.9 %) patients, and erosion in 24 of 34 (70.6 %) patients in the monotherapy group. Typical GI ulcers in patients taking the COX-2-selective inhibitor are shown in Fig. 3. Two of 5 patients with ulcers were prescribed COX-2 non-selective inhibitor and 1 of 5 was COX-2 selective inhibitor before starting this study. Retrospectively, we evaluated the relationship between the prevalence of GI symptoms and ulcers. One of the five patients with GI ulcers was asymptomatic, and the other four patients with ulcers sometimes had stomach ache, stomach distention, or tarry stools. These results show the importance of an endoscopic examination in the diagnosis of GI events because asymptomatic GI complications can exist.

Graham et al. [10] reported that the use of misoprostol prevented NSAID-induced GI complications in patients, as do PPIs. Park et al. [8] reported the preventive efficacy of rebamipide and misoprostol for NSAID-induced peptic ulcer in arthritic patients was not significantly different. These results suggest the importance of PG production in GI management. In an in vitro study, a microarray assay showed that rebamipide upregulated *COX-2* gene expression in the normal rat gastric epithelial cells [11]. It is known that the action of anti-inflammatory agents depends upon reducing COX-2 levels [12]. However, it is necessary to elevate COX-2 in the case of GI injuries to heal them. Present study suggests that the microscopic damage caused by the infiltration of neutrophils into the gastric mucosal cells might already be established by the long-term use of a COX-2-selective inhibitor. The present study also might show that rebamipide exerts a preventive effect in cases of chronic damage by enhancing COX-2 expression in the upper-GI region. Ishihara et al., reported that rebamipide suppressed celecoxib-induced mucosal apoptosis and lesion production in mice, and suppressed celecoxib-induced increases in intracellular Ca^{2+} concentrations and apoptosis in vitro. It also suppressed the increase in the intracellular Ca^{2+} concentration induced by an activator of voltage-dependent L-type Ca^{2+} channels. These results suggest that celecoxib activates voltage-dependent L-type

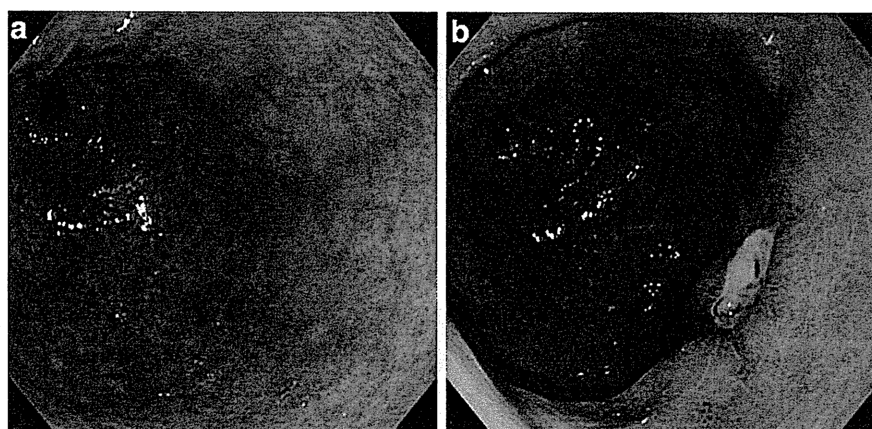

Fig. 3 The representative case of gastric ulcer induced by COX-2-selective inhibitor without rebamipide therapy. A 68-year-old woman with osteoarthritis has no history of GI ulcer. The patient used neither aspirin nor antiplatelet drug. Endoscopic view of her stomach before starting the study has no lesion (**a**). A gastric ulcer was appeared in the prepyloric region after 3 months (**b**)

Ca^{2+} channels and that rebamipide blocks this activation, resulting in the suppression of celecoxib-induced apoptosis [12].

Chan et al. [13] reported that the administration of a COX-2-selective inhibitor plus PPI completely prevented GI bleeding. Although treatment with a PPI is the most useful strategy for the prevention and healing of NSAID-induced upper-GI damage [14], PPIs have some limitations. Based on epidemiological data, the US FDA has warned that the long-term acid suppression of proton pumps induces bone fracture [15]. Moreover, the long-term use of a COX-2-selective inhibitor plus PPI is not cost effective in Japan.

In this study, there were upper-GI ulcers (14.7 %) of patients in the monotherapy group, which is much higher than reported previously. Cheung et al. [7] reported an incidence of 2.8 % for gastroduodenal ulcers during treatment with celecoxib for 3 months. Graham and Chan found that the strongest risk factor for GI complications was the presence of a history of ulcerative complications [16]. Cheung et al. [7] also reported that 1.6 % of a group of patients using celecoxib had a history of gastroduodenal ulcer complications. In our study, 7 of 34 (20.6 %) patients had a history of gastroduodenal ulcer in the monotherapy group, although prevalence of the history of ulcer was not different between the monotherapy group and the combination therapy group. This high frequency might partly explain the high prevalence of upper GI events in the present study. Moreover, the mean age of our patients was > 65 years. Scheiman et al. [17] reported that 16.5 % of patients who received COX-2-selective inhibitors (rofecoxib or celecoxib) developed ulcers within 6 months, as determined with endoscopy. Their results might have been affected by the fact that most of their patients had preexisting GI risk factors, were over 60 years old, or had a history of ulcer in the previous 5 years. Tsuji et al., reported that the administration of a COX-2-selective inhibitor for 4 months induced gastroduodenal ulcer in 8.6 % of a Japanese population, as determined with endoscopy.

Tsuji's report showed a lower prevalence ratio of ulcers compared with the frequency of Scheiman's report.There were more than 70 % of patients that were included in concomitant use of antiulcer drugs in the Tsuji's report [18].

Thus, the risk of the incidence of GI ulcer by using COX-2 selective inhibitor might not be ignored. Therefore, concomitant use of COX-2 selective inhibitor and anti-ulcer drugs might be required in patients with one or more risk factors for GI ulcers, likewise guidelines and consensus meetings are recommended [1, 2].

The limitations of this study were the small sample size and including various diseases. If the absolute difference of GI events between the combination therapy group and the monotherapy group is set at 15 %, the requirement of sample size was calculated to be almost 65 for one arm. A reliable study with greater statistical power is required. Patients with COX-2 non-selective inhibitor and aspirin were included in the present study. It might cause the prevalence of patients with a history of ulcer to be almost 20 % in patients' characteristics. Patients with COX-2 non-selective inhibitor and aspirin should be excluded in a future study.

In conclusion, the administration of a COX-2-selective inhibitor alone induced upper-GI events in 18 % of arthritic patients and erosion in 71 % of them. Rebamipide had a significant effect in preventing these upper-GI events and exacerbation. Our results suggest that rebamipide is a suitable candidate drug for COX-2-selective inhibitor users.

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Conflict of interest None.

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BASIC SCIENCE

Tissue Renin-Angiotensin System in the Intervertebral Disc

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Study Design. Immunohistochemical and biochemical analyses of the rat intervertebral disc (IVD) tissue renin-angiotensin system (tRAS).

Objective. To examine the expression and function of tRAS in the rat IVD.

Summary of Background Data. Angiotensin II (Ang II), the major effector of tRAS, is a hormone that contributes to inflammation and fibrosis in many organs. The expression of tRAS in the rat IVD has not been determined.

Methods. tRAS expression in rat and bovine IVDs was examined using real-time polymerase chain reaction (rat) and immunohistochemistry (rat and bovine). Rat annulus fibrosus cells in monolayer culture were used to examine the biological role of tRAS *in vitro*. The effect of Ang II peptide on extracellular matrix metabolism was assessed by real-time polymerase chain reaction.

Results. mRNA of tRAS components, including angiotensin converting enzyme, Ang II, Ang II receptor type 1, Ang II receptor type 2, and Cathepsin D (a renin-like enzyme), was clearly confirmed by real-time polymerase chain reaction analysis. In rat and bovine annulus fibrosus and nucleus pulposus cells in monolayer culture, immunohistochemical analysis showed that each tRAS component was clearly expressed. In rat IVD tissues, immunoreactivity to each antibody for tRAS components was also observed. Proliferation of rat annulus fibrosus cells was mildly stimulated by Ang II peptide. Ang II peptide also had minor stimulatory effect on the expression of the extracellular matrix components, growth factors, and catabolic proteins.

Conclusion. Our results demonstrate for the first time that the tRAS components necessary to activate tRAS have been found in the normal rat IVD at both mRNA and protein levels. To elucidate the association between tRAS and the process of IVD degeneration, the expression and function of tRAS in the human degenerated IVD should be examined in a future study.

Key words: intervertebral disc, tissue renin-angiotensin system, angiotensin II, IVD degeneration, rat. **Spine 2013;38:E129–E136**

Intervertebral disc (IVD) degeneration is an important clinical problem and one of the underlying factors of low back pain and disability that results in significant medical costs.[1–3] Even in early-stage degeneration, a degenerating IVD can induce low back pain.[3] The progression of degeneration can cause degenerative spinal diseases, such as spinal stenosis, instability, radiculopathy, and myelopathy.[4] Research on the pathogenesis of IVD degeneration is of great importance for the future development of novel IVD therapies, including prophylaxis.

The pathogenesis of IVD degeneration is poorly understood, although biochemical changes typical of the degenerative IVD are known to include a progressive decrease of proteoglycan and collagen type 2 contents with subsequent dehydration and increase of collagen type 1, leading to tissue fibrosis.[5,6] The homeostatic imbalance of matrix metabolism induced by the local production of proinflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor α, and matrix-degrading enzymes, is thought to contribute to the biochemical changes in IVD degeneration.[7–12]

The renin-angiotensin system (RAS) plays a vital role in regulating the physiology of the cardiovascular system. The primary effector molecule of this system, angiotensin II (Ang II), is formed by the enzymatic cleavage of angiotensinogen (AGT) to angiotensin I (Ang I) by renin, with subsequent conversion of Ang I to Ang II by angiotensin-converting enzyme (ACE). Ang II, a classic endocrine hormone that plays a central role in the regulation of blood pressure and volume homeostasis, is the major effector of the RAS. Ang II is also known to have hemodynamics-independent effects, such as participation in inflammation and fibrogenesis in other organs, including the arteries, heart, lung, kidney, and liver.[13]

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TABLE 1. Primers for Real-Time PCR

Genes	Assay ID*	Size (bp)
GAPDH	Rn99999916_s1	87
AGT	Rn00593114_m1	63
Renin	Rn00561847_m1	66
ACE	Rn00561094_m1	90
AT1	Rn01435427_m1	99
AT2	Rn00560677_s1	71
Cat D	Rn00592528_m1	82
Col1	Rn01526721_m1	96
Col2	Rn01637087_m1	97
Agg	Rn00573424_m1	74
TGF-β	Rn99999016_m1	76
IGF-1	Rn00710306_m1	69
MMP-3	Rn00591740_m1	67
MMP-13	Rn01448192_m1	65
IL-1β	Rn00580432_m1	74
ADAMTS-5	Rn01458486_m1	77

**TaqMan Gene Expression Assays (Applied Biosystems).*

Recently, locally produced Ang II has been shown to serve autocrine and/or paracrine functions in several tissues and organs and is called the "tissue renin-angiotensin system (tRAS)."[13] The local production of Ang II by the tRAS has been implicated in the pathogenesis of atherosclerosis and renal fibrosis, as well as in inflammatory diseases, including rheumatoid arthritis.[14] Ang II potentially binds to 2 distinct high affinity plasma membrane receptors, angiotensin II receptor, type 1 (AT1) and angiotensin II receptor, type 2 (AT2). AT1 mediates most physiological actions of Ang II by interacting with various heterotrimeric G proteins.[15,16] These signaling pathways are associated with the production of several growth factors and cytokines (see review by de Gasparo *et al*[17]). Locally produced Ang II from the activation of tRAS also has cell proliferative and proinflammatory properties that modulate the homeostasis of several tissues, as well as contributing to tissue remodeling. However, AT2-receptors are also thought to counteract some of the actions of AT1-receptors. Recently, the expression of tRAS has been reported in the locomotory system, including bone[18–20] and skeletal muscle.[21] To date, the expression of tRAS in IVD tissues is unknown. We hypothesized that tRAS is expressed by IVD cells and may play a role in modulating the metabolism of the IVD.

The purposes of this study were (1) to determine whether IVD cells express the components of tRAS using quantitative real-time polymerase chain reaction (PCR) (rat IVDs) and immunohistochemical analyses (rat and bovine IVDs) and (2) to examine the effect of tRAS activation on cell proliferation and matrix metabolism by rat IVD cells.

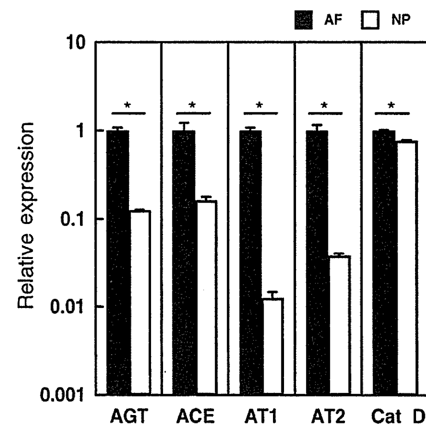

Figure 1. Expression of mRNA for tissue renin-angiotensin system (tRAS) components by rat AF and NP cells. Significantly higher mRNA expression levels of the tRAS components, AGT, ACE, AT1, AT2, and Cat D, are found in AF cells than those in NP cells. *$P < 0.01$. AF indicates annulus fibrosus; NP, nucleus pulposus; AGT, angiotensinogen; ACE, angiotensin-converting enzyme; AT1, angiotensin II receptor, type 1; AT2, angiotensin II receptor, type 2; Cat D, Cathepsin D.

MATERIALS AND METHODS

Cell Isolation

Sixty 12-week-old male Sprague-Dawley rats were used in this study. Lumbar IVDs were harvested from 10 rat spines for each series of the cell culture experiments. The annulus fibrosus (AF) and the nucleus pulposus (NP) were carefully dissected from discs at 6 consecutive levels, L1–L2 to L6–S1. The AF and the NP cells were isolated using sequential enzyme digestion with 0.4% Pronase (Calbiochem, Darmstadt, Germany) for 1.5 hours, followed by 0.025% Collagenase P (Roche, Penzberg, Germany) and 0.001% Deoxyribonuclease II (Sigma-Aldrich, St. Louis, MO) for 3.5 hours at 37°C.[8,22] Following enzyme digestion, the suspension was filtered through 40 μm mesh (Falcon, Franklin Lakes, NJ) for AF cells or 70 μm mesh for NP cells. The filtered cells were washed with Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12; Invitrogen, Grand Island, NY) and the primary culture was started. Isolated cells were cultured in monolayer in 4-chamber slides (BD Biosciences, Bedford, MA) or 6-well tissue culture plates (CellStar, Greiner Bio-One, Frickenhausen, Germany) at 4.0×10^4 cells/mL with 5% CO_2, 95% air in complete medium (DMEM/F12 containing 10% fetal bovine serum [FBS; CELLect, MP Biomedicals Inc., Germany], 25 μg/mL ascorbic acid [Sigma-Aldrich], and 50 μg/mL gentamicin [Invitrogen]). The medium was changed every second day. For immunohistochemical analysis, bovine IVD cells were isolated from 15- to 18-month-old bovine tail discs. The cells were cultured in monolayer in the same way as rat IVD cells. Phase-contrast micrographs of monolayer-cultured IVD cells showed that cells isolated from the NP tissues were large and vacuolated, whereas cells cultured from the AF were polygonal and fibroblastic. Because similar observations

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have been previously reported,[23] we were able to morphologically identify the cells isolated from rat IVD tissues as AF or NP cells.

Because of the difficulty in culturing a substantial number of notochordal-like cells from rat NP tissues, AF cells were used for the functional analysis of Ang II. Notochordal cells are not present in adult human IVD tissues[24]; therefore, we decided not to use rat notochordal cells for the Ang II treatment studies.

RNA Isolation

Total RNA was isolated from rat AF and NP cells in monolayer culture using Isogen (NipponGene, Toyama, Japan), according to the manufacturer's instructions. Total RNA was reverse-transcribed using the first-strand cDNA synthesis kit (Roche Applied Science, Mannheim, Germany) with the DNA thermal cycler (Veriti; Applied Biosystems, Foster City, CA), according to the manufacturer's protocol.

Detection of mRNA of tRAS Components Using Quantitative Real-time PCR

Total RNA of the rat AF and NP cells was isolated after the cells were initially cultured in monolayer for 7 days. The expression levels of the tRAS components were quantified using real-time PCR with TaqMan Gene Expression Assays (Applied Biosystems), using the primer pairs for TaqMan genomic assays. The assay was calibrated using 87bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (TaqMan Gene Expression Assay) as an internal control. To determine the expression of tRAS components, the resultant cDNA (3 replicates) was amplified for the target genes *AGT*, *renin*, *ACE*, *AT1*, *AT2* and *Cathepsin D* (Table 1). The cycle used a 15-second denaturation at 95°C and a 1-minute annealing and extension at 60°C, using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The relative expression of the tRAS components was calculated using the comparative threshold (Ct) method.[25]

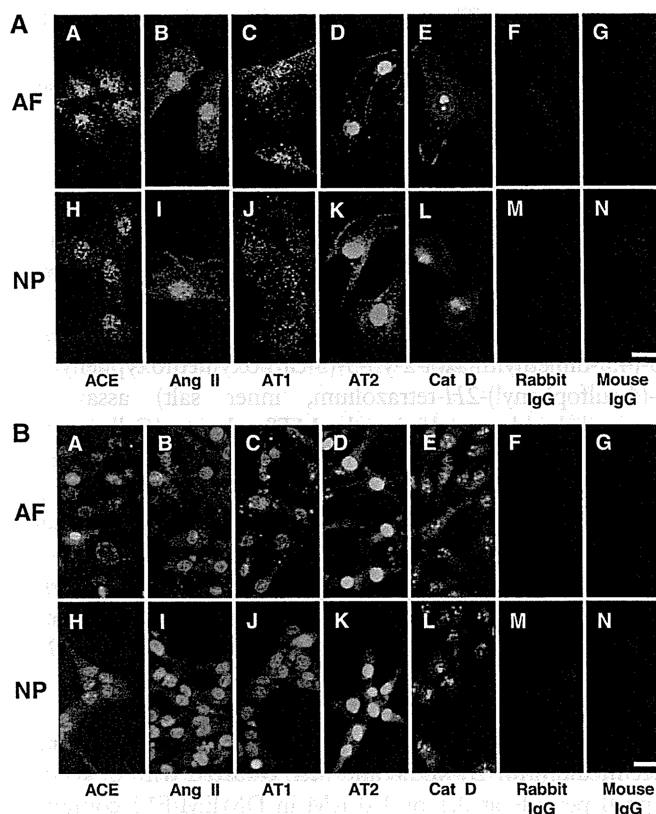

Figure 2. Immunohistochemical staining for tissue renin-angiotensin system (tRAS) components and Cathepsin D in cultured AF and NP cells. AF and NP cells isolated from rat **(A)** and bovine **(B)** intervertebral discs were cultured in monolayer for 7 days (AF: **A–G**; NP: **H–N**). Rabbit IgG (**F, M**) and mouse IgG (**G, N**) were used as isotype controls. Rat AF and NP cells were observed using confocal microscopy **(A)**. Bovine AF and NP cells were observed using fluorescence microscopy **(B)**. Immunoreactivity (green) is clearly seen in the AF and NP cells. Nuclei are stained with propidium iodide (red). Scale bar: 20 μm. AF indicates annulus fibrosus; NP, nucleus pulposus; ACE, angiotensin-converting enzyme; Ang II, *angiotensin* II; AT1, angiotensin II receptor, type 1; AT2, angiotensin II receptor, type 2; Cat D: Cathepsin D; IgG, immunoglobulin G.

Immunohistochemistry of IVD Cells

AF and NP cells isolated from both rat and bovine discs were monolayer-cultured on chamber slides for 5 days. The cells were then fixed in 4% paraformaldehyde, treated with a blocking solution containing 10% skim milk, and permeabilized in 0.1% Triton X (NACALAI TESQUE Inc., Kyoto, Japan). The cells were then incubated with anti-ACE (sc-23908, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Ang II (T-4007, 1:200; Peninsula Laboratories, San Carlos, CA), anti-AT1 (sc-1173, 1:200; Santa Cruz Biotechnology), anti-AT2 (sc-9040, 1:200; Santa Cruz Biotechnology), or anti-Cathepsin D (sc-10725, 1:200; Santa Cruz Biotechnology) antibodies overnight at 4°C. A secondary Alexa 488-conjugated anti-rabbit immunoglobulin G (IgG) (1:200; Molecular Probes, Eugene, OR) or anti-mouse IgG (1:200; Molecular Probes) antibody was applied for 3 hours. The nuclei were stained with propidium iodide (1:100; Molecular Probes) for 2 minutes and cover-slipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Rat samples were imaged using confocal microscopy (Fluoview FV1000; Olympus, Tokyo, Japan). Bovine samples were imaged using fluorescent microscopy. Rabbit IgG or mouse IgG was used as isotype controls.

Immunohistochemistry of IVD Tissues

Rat lumbar spines (L1–L6) were removed and fixed in 4% paraformaldehyde, followed by decalcification in 30% ethylenediaminetetra acetic acid for 28 days. The samples were then embedded in paraffin and serial 5-μm sections adjacent to the sections stained with safranin-O were used for immunohistochemical analysis. After blocking endogenous peroxidase activity, sections were heated with 0.01 M of citrated buffer (pH 6.0) and incubated overnight at 4°C with the primary antibodies used in the monolayer culture study described in the previous section. The secondary staining was processed for immunohistochemistry as also described in the

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previous section. The appropriate rabbit IgG or mouse IgG was used as the isotype control. For positive controls, blood vessels (aorta) were removed and processed for immunohistochemical analysis.

Cell Proliferation

The AF cells (at 5.0×10^3 cells/well) were cultured in serum-free medium for 24 hours and then cultured with or without Ang II peptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe; Peptide Institute, Osaka, Japan) at 0.1, 1.0, or 10 μM in DMEM/F12 + 0.3% FBS + 50 μg/mL gentamicin in 96-well plates. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt) assay was accomplished by combining the MTS solution (Cell titer 96R AQueous Assay; Promega, Madison, WI) with phenazine methosulfate (PMS) solution in the ratio 20:1. On days 2 and 7 (n = 5, respectively), 20 μL of MTS/PMS was added to each cell-containing well and incubated for 3 hours at 37°C in a humidified 5% CO_2 atmosphere. Subsequently, the absorbance was measured at 492 nm using a spectrophotometric plate reader (Multiskan JX; ThermoFisher Scientific, Ottawa, ON).

Real time-PCR Analysis of Matrix Metabolism

After 7 days of preculture, the AF cells were cultured in serum-free medium for 24 hours and then cultured with or without Ang II peptide at 0.1 or 1.0 μM in DMEM/F12 containing 0.3% FBS for an additional 24 hours (n = 6). Total RNA of the AF cells treated with Ang II peptide was isolated as described previously. After treatment with Ang II peptide, the resultant cDNA (in triplicate) was amplified for the following target genes: collagen type 1 (*Col1*), collagen type 2 (*Col2*), aggrecan (*Agg*), transforming growth factor-β (*TGF-β*), insulin like growth factor-1 (*IGF-1*), matrix metalloproteinase-3 (*MMP-3*), *MMP-13*, *IL-1β*, and *a d*isintegrin *a*nd metalloproteinase with thrombospondin motifs 5 (*ADAMTS-5*) (Table 1).

Statistical Analysis

The data are expressed as the mean ± standard deviation of 5 replicate determinations from 6 separate experiments for the cell proliferation assay. For quantitative PCR analyses, the results of assays of 3 replicates of 6 separate cultures were statistically analyzed. One-way analysis of variance was used to assess the effects of culture conditions on the results. The *post hoc* analyses were performed using the Fisher PLSD test. The evaluation of statistical differences between the groups was determined using the unpaired Student *t* test. Significance was accepted at $P < 0.05$.

RESULTS

mRNA Expression of tRAS Components by Rat IVD Cells

The expression levels of the components of tRAS were quantified using real-time PCR. mRNA expression levels of the tRAS components, including AGT, ACE, AT1, and AT2, were clearly identified by both AF and NP cells. However, no expression of renin was found. Alternatively, the expression of Cathepsin D (a tissue renin-like enzyme) was clearly identified. Significantly higher mRNA expression levels of the tRAS components, AGT, ACE, AT1, AT2, and Cathepsin D, were found in AF cells than those in NP cells ($P < 0.01$) (Figure 1).

Fluorescent Immunohistochemical Analysis of Intervertebral Disc Cells and Tissues

In rat AF and NP cells in monolayer culture, immunoreactivity to ACE, Ang II, AT1, AT2, and Cathepsin D was clearly identified (Figure 2A). Confocal images revealed that immunoreactivity to ACE and Ang II was found in granules in the cytoplasm in both AF and NP cells. AT1 was primarily found in cytoplasm and AT2 in cell membranes of both AF and NP cells. Similar to rat IVD cells, immunoreactivity to ACE, Ang II, AT1, AT2, and Cathepsin D was clearly identified in bovine AF and NP cells (Figure 2B). No immunoreactivity was found in the isotype controls.

Immunoreactivity to Ang II, AT1, and AT2 was clearly seen in the endothelial layer of the rat blood vessel (aorta) (positive control tissue) (Figure 3P–R). In rat IVD tissues, immunoreactivity to ACE, Ang II, AT1, AT2, and Cathepsin D was also identified. All components with tRAS immunoreactivity were localized on fibrochondrocytes that were aligned longitudinally between the collagen fiber bundles in the anterior AF (Figure 3A, D, G, J, M) and posterior AF (Figure 3C, F, I, L, O). In the NP, immunoreactivity of all the components of tRAS was localized in the cytoplasm and/or cell membrane of propidium-positive cells (Figure 3B, E, H, K, and N). No immunoreactivity was found in the isotype controls (Figure 3S).

Cell Proliferation by Rat IVD Cells

The effect of Ang II peptide on cell proliferation was measured using the MTS assay. Stimulation of rat AF cells with Ang II peptide significantly, but mildly, increased the proliferation of rat IVD cells (% of control: [day 2]: 0.1 μM Ang II: 0%; 1 μM Ang II: 6%, $P < 0.01$; 10 μM Ang II: +17%, $P < 0.01$; [day 7]: 0.1 μM Ang II: +6%; 1.0 μM Ang II: +11%, $P < 0.01$; 10 μM Ang II: +17%, $P < 0.01$; all *vs.* control group) (Figure 4).

Effect of Ang II Peptide on Matrix Synthesis by Rat IVD Cells

The mRNA expression levels of Col1, Col2, and Agg in rat AF cells were quantified using real-time PCR. The stimulation of these cells with Ang II peptide at both 0.1 μM or 1.0 μM significantly, but mildly, increased the mRNA expression of Col1, Col2, and Agg above those of the control group (% of control: [0.1 μM Ang II] Col1: +25%, $P < 0.05$; Col2: +54%, $P < 0.01$; Agg: +20%, $P < 0.01$; [1.0 μM Ang II] Col1: +50%, $P < 0.01$; Col2: +68%, $P < 0.01$; Agg: +50%, $P < 0.01$, all *vs.* control) (Figure 5).

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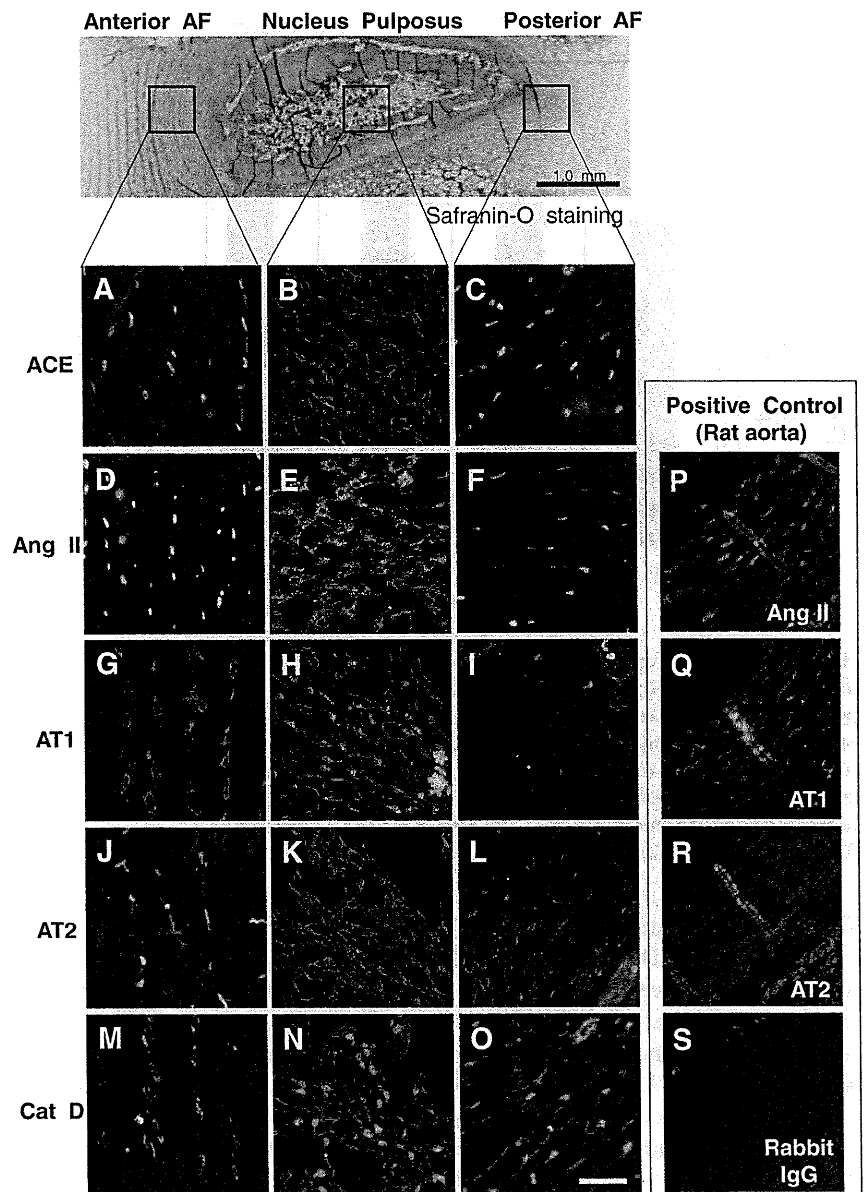

Figure 3. Immunohistochemical staining for tissue renin-angiotensin system (tRAS) components and Cathepsin D in rat intervertebral disc (IVD) tissues. IVD tissues visualized using fluorescence microscopy. Immunoreactivity to ACE, Ang II, AT1, AT2, and Cathepsin D is clearly seen in AF and NP tissues (**A–O**). Immunoreactivity to Ang II, AT1, and AT2 of rat blood vessel tissues (aorta) was used as positive controls (**P–R**). Rabbit IgG was used for the isotype control (**S**). Scale bar: 250 μm. AF indicates annulus fibrosus; NP, nucleus pulposus; ACE, angiotensin-converting enzyme; Ang II, angiotensin II; AT1, angiotensin II receptor, type 1; AT2, angiotensin II receptor, type 2; Cat D, Cathepsin D; IgG, immunoglobulin G.

Effect of Ang II Peptide on the Expression of Anabolic and Catabolic Factors by Rat IVD Cells

The mRNA expression levels of IGF-1, TGF-β, IL-1β, MMP-3, MMP-13, and ADAMTS-5 in rat AF cells were quantified using real-time PCR (Figure 6). Stimulation of rat IVD cells with Ang II peptide at 0.1 μM or 1.0 μM showed a significant, but minor, upregulation of mRNA expression of IGF-1, TGF-β, and ADAMTS-5 compared with those of the control group (% increase: [0.1 μM Ang II] TGF-β, +13%, $P < 0.01$; IGF-1, +39%, $P < 0.01$; ADAMTS-5, +31%, $P < 0.01$ [1.0 μM Ang II] TGF-β, +18%, $P < 0.01$; IGF-1, +42%, $P < 0.01$; ADAMTS-5, +70%, $P < 0.01$). The mRNA expression of IL-1β, MMP-3, and MMP-13 was not significantly changed by the stimulation of Ang II peptide.

DISCUSSION

The expression of tRAS has been found in several organs and tissues, including heart, vasculature, central and peripheral nervous systems, skin, digestive organs, sensory organs, lymphatic tissue, and adipose tissue (see review by Paul *et al*[13]). The expression of tRAS was also recently reported in bone tissue[18–20]; however, its expression in nonvascular tissues, such as the IVD, remains unknown. Our results show that the mRNA expression of tRAS components (AGT, ACE, AT1, and AT2) was clearly identifiable in rat IVD cells; however, no significant mRNA expression of renin was detected. In lieu of renin, tissue renin-like enzymes, represented by serine-proteases, such as Cathepsin D and G, are known to catalyze the formation of Ang I.[26] These renin-like enzymes are found in both normal and degenerated human IVD tissues.[27–29]

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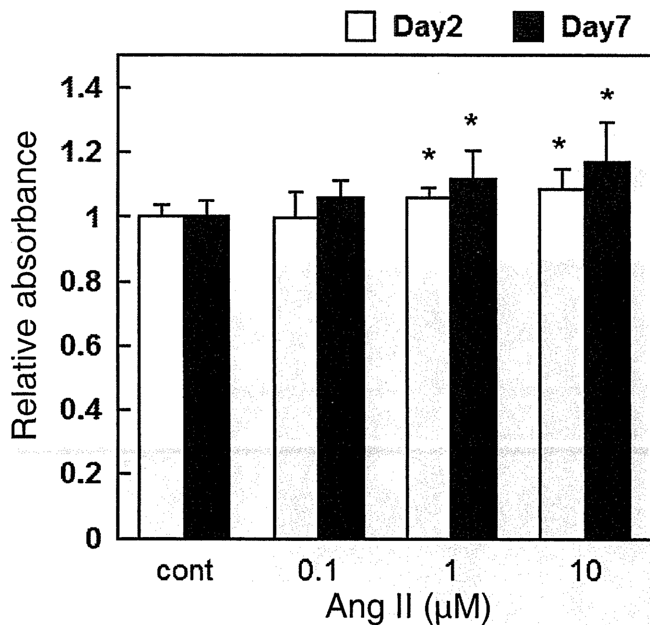

Figure 4. Cell proliferation of rat intervertebral disc (IVD) cells after treatment with Ang II peptide. The effects of Ang II peptide, on cell proliferation by rat annulus fibrosus (AF) cells was measured using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay. Stimulation of rat AF cells with Ang II peptide at 1.0 and 10 μM significantly, but mildly, upregulates the proliferation of rat IVD cells on days 2 and 7. $*P < 0.01$ *versus* control. Ang II indicates angiotensin II.

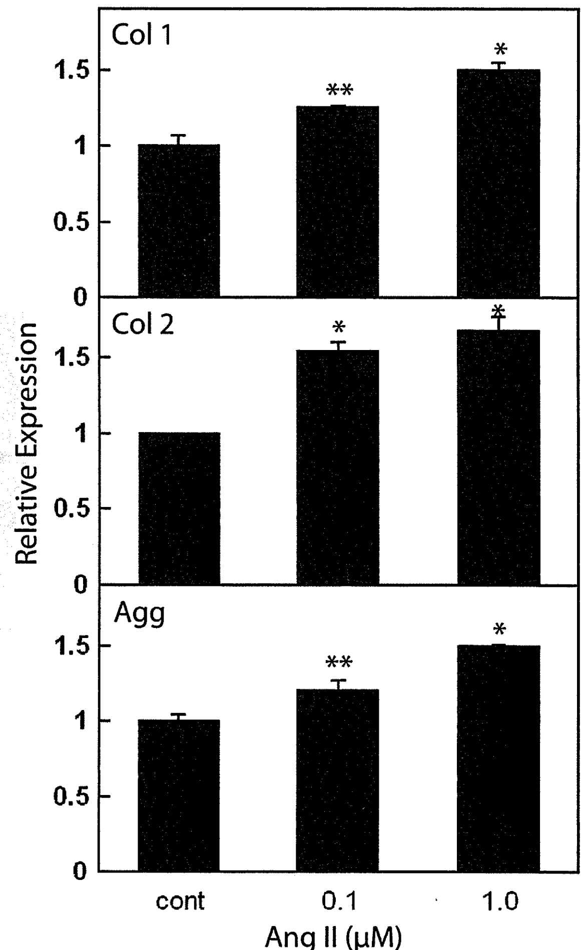

Figure 5. Effect of Ang II peptide on mRNA expression of extracellular matrix components by rat intervertebral disc cells. The mRNA expression levels of Col1, Col2, and Agg by annulus fibrosus (AF) cells were quantified by real-time polymerase chain reaction. Stimulation of rat AF cells with Ang II peptide at 0.1 μM and 1.0 μM significantly increases mRNA expression of Col1, Col2, and Agg in a dose-dependent manner compared with the control group. $*P < 0.01$, $**P < 0.05$ *versus* control. Col1 indicates collagen type 1; Col2, collagen type; Agg, aggrecan; Ang II, angiotensin II.

Therefore, as an alternative to renin, we examined the expression of Cathepsin D by rat IVD cells and tissues. The results of our study showed that the expression of Cathepsin D was clearly identified at both mRNA and protein levels. Thus, theoretically, tRAS can be activated in an autocrine or paracrine manner within IVD tissues.

The phenotype of IVD cells has been shown to change according to culture conditions.[30,31] It has been reported that the gene expression of collagen types 2 and 10, and aggrecan was significantly decreased in monolayer layer cultures of human AF and NP cells.[30] To minimize the effects of monolayer culture conditions, primary cultured rat AF and NP cells were used for this study. Because a previous report showed significant changes in the phenotype of human disc cells after 10 days' culture in monolayer,[30] rat IVD cells were cultured in monolayer for only 7 days before isolating total RNA to assess the gene expression of tRAS components.

A quantitative mRNA analysis found significantly higher mRNA expression levels of AGT, ACE, AT1, AT2, and the renin-like enzyme Cathepsin D in AF cells than those in NP cells, suggesting that these tRAS components are more highly expressed in fibrochondrocytic cells of the AF than in notochordal-like cells of the NP. Immunohistological analysis found clear indications that tRAS components were expressed in rat IVD cells and tissues.

Rats retain notochordal cells in the mature adult NP. However, adult human NP tissues do not contain notochordal cells[24]; they have been replaced by chondrocyte-like cells. To more closely reflect the situation of adult human IVD tissues, we have also examined the expression of tRAS components in IVD cells from the bovine, a chondrodystrophoid animal whose IVDs are similar to the human IVD. We have examined the immunoreactivity to the components of tRAS by bovine AF and NP cells. Immunoreactivity to tRAS components was clearly found in both bovine AF and NP cells. The results of this additional study suggest the possibility that tRAS exists and functions in the chondrodystrophoid IVD, including the human IVD.

Consistent with previous reports,[32,33] our results show that the cell proliferative activity of rat AF cells was significantly, but mildly, upregulated by treatment with Ang II peptide in a dose-dependent manner.

Under stimulation by the Ang II peptide, the mRNA expression of collagen types 1 and 2, and aggrecan (the major large proteoglycan) was slightly upregulated by rat AF cells.

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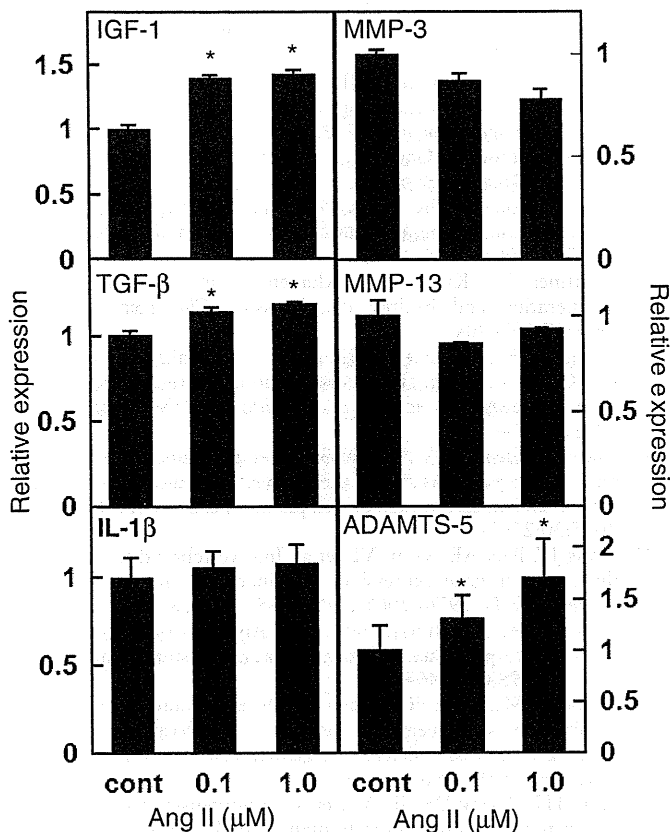

Figure 6. Effect of Ang II peptide on mRNA expression of growth factors and catabolic enzymes by rat intervertebral disc (IVD) cells. The mRNA expression levels of IGF-1 and TGF-β, MMP-3, MMP-13, IL-1β, and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5) in rat annulus fibrosus cells were quantified by real-time polymerase chain reaction. Stimulation of rat IVD cells with Ang II peptide at 0.1 μM and 1.0 μM did not significantly change the mRNA expression of IL-1β, MMP-3, and MMP-13 compared with the control group. However mRNA expression of ADAMTS-5 significantly increases with stimulation by Ang II peptide. *$P < 0.01$ *versus* control. IGF-1 indicates insulin-like growth factor-1; MMP-3, matrix metalloproteinase-3; TGF-β, transforming growth factor-β; IL-1β, interleukin-1β; ADAMTS-5, a disintegrin and metalloproteinase with thrombospondin motifs 5; Ang II, angiotensin II.

Our results suggest the possibility that Ang II has a minor potential to influence IVD matrix production. Similar to our results, Ang II was shown to induce the expression of collagen type 1, a major component of skin, through a TGF-β1-dependent pathway in human dermal fibroblasts.[34] Gao *et al*[35] reported similar findings for cardiac fibroblasts. In vascular smooth muscle cells, Ang II increased the content of small proteoglycans, such as biglycan and perlican, which bind to low-density lipoprotein, thus contributing to the development of atherosclerosis.[36,37]

Proinflammatory cytokines, such as IL-1β and tumor necrosis factor α, whose expression is upregulated in degenerated IVD tissue, are thought to contribute to the progression of IVD degeneration by inducing such matrix-degrading enzymes as the matrix metalloproteinases (MMPs) and ADAMTS.[9,10,12,38,39] Our results indicate that the mRNA expression of ADAMTS-5, which degrades collagen type II, was slightly stimulated by Ang II peptide. However, other catabolic factors, IL-1β, MMP-3, and MMP-13, showed no significant changes with stimulus by Ang II.

Local RAS has diverse actions in many organs. In recent years, accumulating evidence suggests a novel concept that locally or tissue-based RAS has a pivotal role in modulating tissue homeostasis. However, it has been proposed that changes in the activity of RAS within the microenvironment induce pathophysiological stimuli shown to be related to subacute or chronic diseases, such as hypertension, heart failure, atherosclerosis, and diabetic nephropathy.[13] Unlike cardiovascular organs or tissues, the IVD is avascular. Within IVD tissues relatively small numbers of cells are interspersed within an abundant extracellular matrix. Our study showed that tRAS exists in normal rat IVD tissues. However, unlike the microenvironment of vascular tissues, tRAS has a minor potential to affect the matrix metabolism within normal IVD tissue.

To elucidate the association between the expression of tRAS and IVD degeneration, future studies are needed to clarify the function of tRAS in pathological conditions and differences in expression of tRAS in normal and degenerated human IVD tissues.

➢ Key Points

- ❑ mRNA of tRAS was expressed by rat IVD cells.
- ❑ tRAS components in rat and bovine IVD cells and rat IVD tissues were identified using immunohistochemical analysis.
- ❑ Cell proliferation was mildly upregulated by Ang II peptide in cultured rat IVD cells.
- ❑ The expression of extracellular matrix, growth factors, and a matrix-degrading enzyme by rat IVD cells was mildly stimulated by Ang II peptide.

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