

# Upregulation of Kallistatin Expression in Rheumatoid Joints

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**ABSTRACT. Objective.** Previous studies demonstrated suppression of rat ankle arthritis by local injection of kallistatin gene, a negative regulator of angiogenesis. We analyzed circulating levels, synovial concentrations, and tissue localizations of kallistatin in patients with rheumatoid arthritis (RA).

**Methods.** Paired plasma and joint fluid samples were simultaneously obtained from 24 patients with RA and 14 with osteoarthritis (OA). Synovial tissues from 5 patients with RA and 5 with OA were obtained during surgery. Fibroblast-like synoviocytes (FLS) and mononuclear cells (MNC) were prepared. ELISA was used to measure kallistatin levels of plasma, joint fluid, cell lysate, and synovium homogenate extract. Synovial tissues were subjected to Western blot and immunohistochemical staining. In addition, the tissue kallikrein (TK) levels of plasma and joint fluid samples were also measured by the ELISA.

**Results.** Circulating and synovial levels of kallistatin and TK were elevated in patients with RA. The immunohistochemical assay exhibited stainings of kallistatin on both infiltrating MNC and FLS. Intracellular kallistatin levels were significantly elevated in MNC and FLS from patients with RA.

**Conclusion.** Elevated kallistatin levels were demonstrated in patients with RA, particularly in synovial tissues, FLS, and MNC. This report is the first to demonstrate upregulation of kallistatin expression in rheumatoid joints. (First Release Oct 15 2007; J Rheumatol 2007;34:2171-6)

*Key Indexing Terms:*

KALLISTATIN

RHEUMATOID ARTHRITIS

ANGIOGENESIS

Greater understanding of the etiology of rheumatoid arthritis (RA) has identified molecular targets for the development of therapeutic candidates. The formation of hypertrophied synovium (known as pannus) is the primary feature, thus a therapeutic approach is to limit its size by interfering with the blood supply<sup>1</sup>. Studies of angiogenesis inhibitors in animal arthritis support the hypothesis that suppression of new blood vessel formation can ameliorate joint inflammation. Tissue kallikrein (TK) is a serine protease of physiological importance contributing to the regulation of inflammatory responses<sup>2</sup>. It releases kinins by the cleavage of the kininogen substrates and processes a variety of promolecules

including matrix metalloproteinase<sup>3</sup>. Serine proteinase inhibitors (serpins) are involved in the antiangiogenesis activity<sup>4,5</sup>. Kallistatin, a unique serpin, functions as an endogenous TK-binding protein and inhibitor<sup>5</sup>. TK gene delivery or protein infusion has been shown to promote *in vivo* angiogenesis<sup>6</sup>.

Previous studies demonstrated that the antiangiogenesis response played a crucial role in suppression of arthritis by kallistatin gene therapy<sup>7</sup>. We analyzed circulating levels, synovial concentrations, and tissue localizations of kallistatin in patients with RA. Cells responsible for production of kallistatin within rheumatoid joints were also identified.

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*Supported by NSC 94-2314-B-006-052 and NSC 95-2314-B-006-010 from the National Science Council, Taiwan, and by NIH HL-44083 from the National Institutes of Health, USA.*

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*Accepted for publication July 27, 2007.*

## MATERIALS AND METHODS

**Patients.** Venous plasma samples and joint fluid specimens from knees were simultaneously obtained from 24 patients with RA (18 women, 6 men, ages 30–79 yrs, mean 58.1 ± 13.6). Fourteen patients with osteoarthritis (OA; 8 women, 6 men, ages 54–83 yrs, mean 64.6 ± 8.5) were also recruited as a control group. Diagnoses of RA and OA were according to the criteria of the American College of Rheumatology<sup>8,9</sup>. In patients with RA, there were 18 with seropositivity and 10 had extraarticular clinical manifestations. Disease duration varied from 1.5 to 19 years (mean 7.2 ± 4.1). Erythrocyte sedimentation rates (ESR) varied from 3 to 122 mm/h (mean 27.5 ± 29.4) during the collection of clinical samples. The disease duration of patients with OA varied from 1.5 to 20 years (mean 6.1 ± 5.3). These patients were regularly followed up at the Outpatient Department of National Cheng Kung University Hospital, a national medical center located in an area of southern Taiwan with more than 7 million inhabitants. Synovial tissues were obtained from 5 patients with RA and 5 with OA during orthopedic surgery. The Ethics Committee of the National Cheng Kung University Hospital approved our study. Informed consent was obtained from each study participant.

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*Preparation of fibroblast-like synoviocytes (FLS) and mononuclear cells (MNC).* Freshly prepared synovial tissues were cultured in Dulbecco's modified Eagle's medium with 2 mM L-glutamine, 20 mM HEPES buffer, 10% fetal calf serum, and antibiotics in Petri dishes in a humidified 5% CO<sub>2</sub> incubator. After cell adherence for 48 h, nonadherent cells were removed and adherent cells were cultured continuously until confluence. FLS became a homogeneous population after more than 3 passages. MNC were isolated by Ficoll density-gradient centrifugation. Both FLS and MNC were washed 3 times with Hanks' balanced salt solution medium and further subjected to the cell lysis buffer, and were stored at -70°C until use.

*Kallistatin and TK ELISA.* The kallistatin ELISA was performed as described<sup>10</sup>. In brief, standards and samples (plasma, synovial fluid, cell lysate, or joint homogenate extract) were added at appropriate dilutions and in duplicate into 96-well microtiter plates coated with nonlabeled antibodies. The TK ELISA was carried out with similar methods.

*Preparation of joint homogenate extracts.* Synovial tissues obtained from surgery were cut into small pieces, and were further homogenized in homogenization buffer containing protease inhibitor cocktail (Halt; Pierce, Rockford, IL, USA). After the homogenization was completed, homogenates were centrifuged at 2000 g for 10 min and filtered through a 0.45 µm filter (Millipore, Bedford, MA, USA). Samples were stored at -70°C until use.

*Kallistatin Western blot.* Joint homogenate extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels at 100 V for 3 h, and then transferred to nitrocellulose membranes at 90 V for 2 h at 4°C. The membrane was blocked at 4°C overnight with the 5% nonfat milk in TBS-0.1% Tween 20 buffer (TBS-T), washed with TBS-T, and further incubated with anti-kallistatin antibodies at appropriate dilutions for 2 h at room temperature. The membrane was washed with TBS-T and incubated for 2 h with appropriately diluted peroxidase-conjugated anti-mouse IgG. Following washing, the blot was then developed with the enhanced chemiluminescence system. β-actin was used as the loading control.

*Kallistatin immunohistochemical staining.* The synovial tissues were embedded in OCT compound and cryostatic sections (5 µm) were prepared. Frozen sections were stained with anti-kallistatin monoclonal antibodies and were further incubated with peroxidase-labeled anti-mouse IgG antibodies. The staining was finally developed by adding chromogen substrate, and the slides were counterstained with hematoxylin.

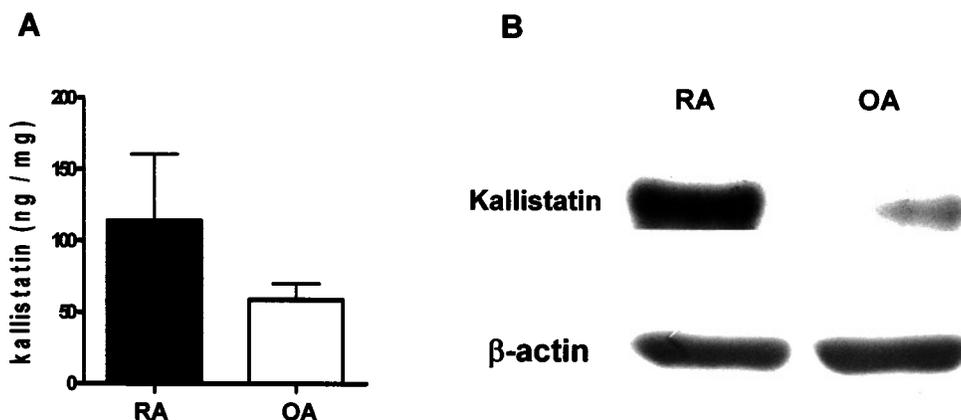
*Statistical analyses.* Data were expressed as mean ± standard deviation. The Mann-Whitney rank-sum test was carried out for comparisons between patients with RA and those with OA, and between different groups of patients with RA. The coefficient of correlation (γ) for linear regression was calculated for 2 different measures from patients with RA. The p value < 0.05 was considered to be significant.

## RESULTS

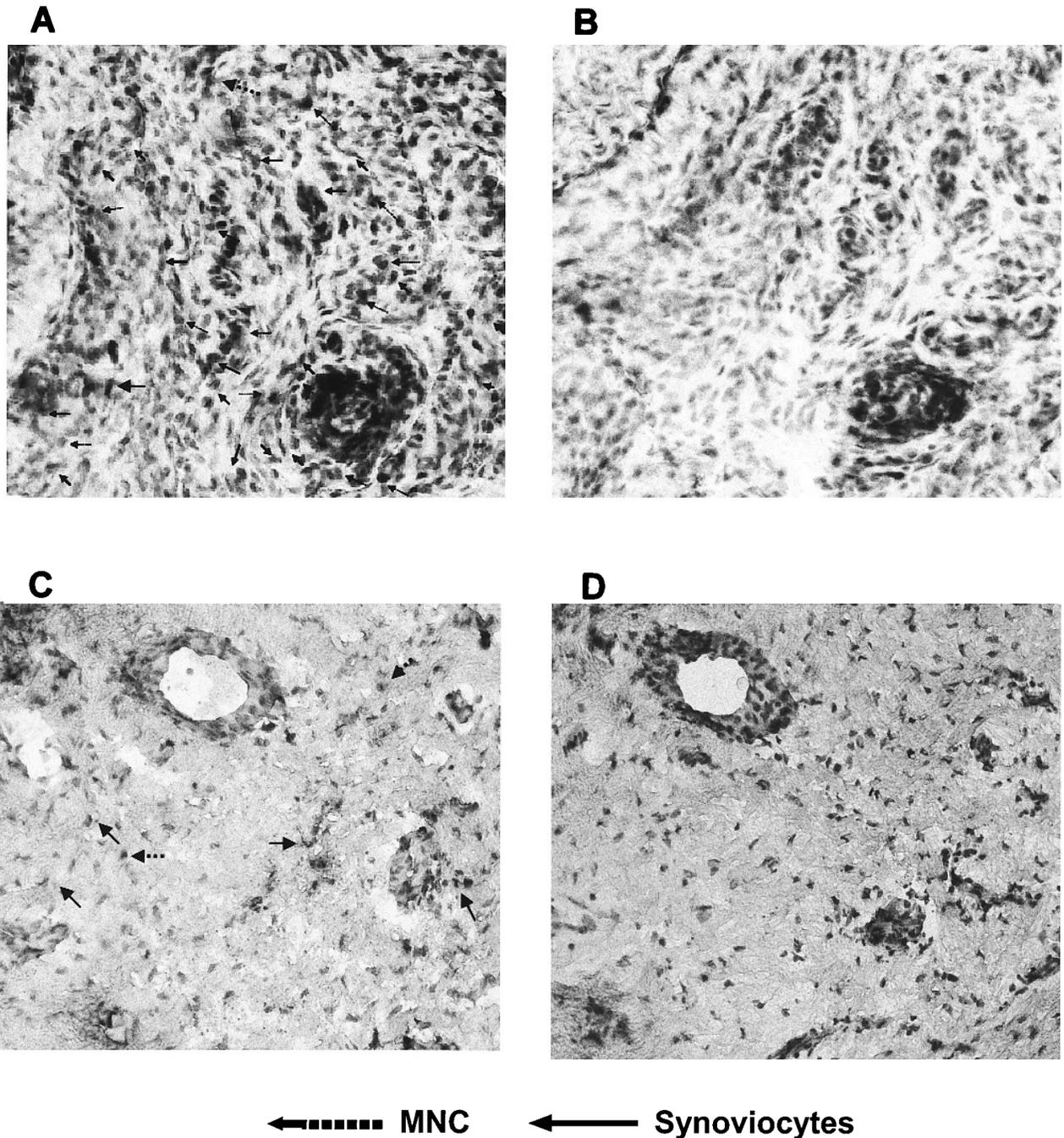
*Kallistatin in synovial tissues.* Kallistatin levels were higher in RA joint homogenate extracts as compared with OA (Figure 1A, 113.8 ± 46.8 vs 58.8 ± 11.1 ng/mg protein; p < 0.005). Western blot analyses of the extracts demonstrated a ~53 kDa band more intensely in RA (Figure 1B). Figure 2 shows expressions of kallistatin on synoviocytes and infiltrating MNC within synovial tissues from patients with RA and OA and control sections receiving only secondary antibodies (Figure 2A and 2B for RA, Figure 2C and 2D for OA).

*Kallistatin in synovial fluid, plasma, and cell lysate samples.* Kallistatin levels were increased in synovial fluid from RA as compared with OA (Figure 3A, 14.3 ± 7.3 vs 7.0 ± 1.8 µg/ml; p < 0.01). Circulating levels of kallistatin in plasma were elevated in RA as compared with OA (Figure 3B, 26.9 ± 13.5 vs 16.8 ± 3.9 µg/ml; p < 0.05). Kallistatin levels were higher in plasma than in joint fluid from either RA or OA (p < 0.001). In addition, TK levels were increased in plasma and synovial fluid from RA as compared with OA (plasma, 8.1 ± 5.0 vs 2.9 ± 2.2 ng/ml, p < 0.001; synovial fluid, 9.4 ± 4.8 vs 1.6 ± 2.0 ng/ml, p < 0.001). Since the stainings of kallistatin were localized on infiltrating MNC and synoviocytes, we analyzed their intracellular levels further. Higher levels were detected in MNC from RA as compared with OA (Figure 3C, 6.2 ± 1.7 vs 2.8 ± 1.0 ng/mg protein; p < 0.001). Intracellular levels in FLS were increased in RA as compared with OA (Figure 3D, 1.5 ± 0.1 vs 0.68 ± 0.02 ng/mg protein; p < 0.05). Additionally, we examined if the synovial fluid and plasma levels of kallistatin or TK were associated with clinical or laboratory features of patients with RA. However, we found no statistical association of kallistatin or TK levels with the presence or absence of rheumatoid factor or extraarticular manifestations (data not shown).

*TK to kallistatin ratios in synovial fluid and plasma samples.* We further analyzed the TK to kallistatin ratios in synovial fluid and plasma samples in order to measure how much the TK/kallistatin system is upregulated in patients with RA in



**Figure 1.** Kallistatin in synovial tissues from patients with RA and OA. A. Kallistatin levels in homogenate extracts (RA or OA, n = 5), RA vs OA, p < 0.005. B. Representative Western blot shows 53 kDa kallistatin bands and β-actin bands as control.



**Figure 2.** Immunohistochemical staining of kallistatin on synovial tissues. A. Expression of kallistatin on MNC (broken-line arrows) and synoviocytes (solid arrows) in RA synovium. Dense staining of kallistatin was observed on lymphocyte aggregates (lower right corner). B. Control RA synovial section receiving only anti-mouse IgG. C. Expression of kallistatin in OA synovium. Faint staining of kallistatin was observed on endothelial cells around a blood vessel (top). D. Control OA synovial section receiving only anti-mouse IgG. Original magnification 400 $\times$  for all.

respect to patients with OA. TK to kallistatin ratios were significantly higher in both synovial fluid and plasma samples from RA as compared with OA (Figure 4A, synovial fluid,  $7.0 \times 10^{-4} \pm 1.7 \times 10^{-4}$  vs  $1.9 \times 10^{-4} \pm 1.8 \times 10^{-4}$ ,  $p < 0.001$ ; Figure 4B, plasma,  $3.0 \times 10^{-4} \pm 0.6 \times 10^{-4}$  vs  $1.6 \times 10^{-4} \pm 0.7 \times 10^{-4}$ ,  $p < 0.001$ ). Moreover, the elevated levels of TK were signifi-

cantly associated with increased concentrations of kallistatin, a possible counter-regulation effect on the enhanced angiogenesis induced by elevated TK levels within the rheumatoid joints (Figure 4C, synovial fluid,  $\gamma = 0.964$ ,  $p < 0.001$ ; Figure 4D, plasma,  $\gamma = 0.960$ ,  $p < 0.001$ ). In addition, there was a significant correlation between the ESR values and the kallistatin

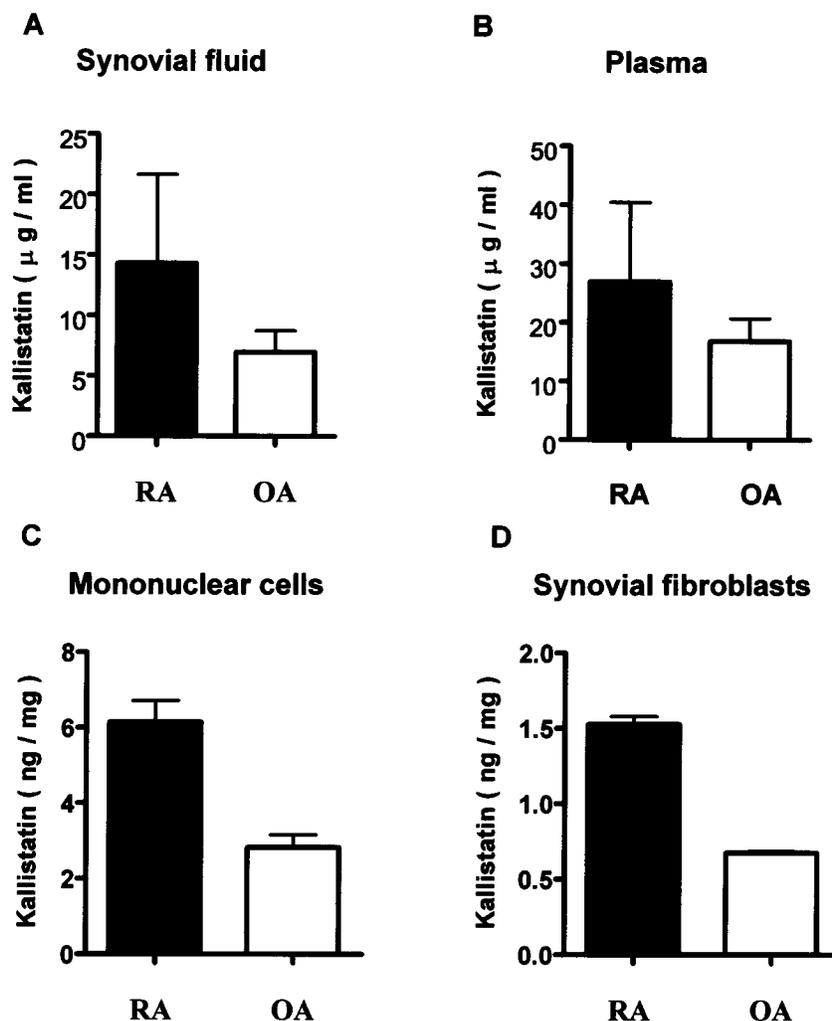


Figure 3. Kallistatin in synovial fluid, plasma and cell lysate samples. A. Synovial fluid (RA, n = 24; OA, n = 14), RA vs OA,  $p < 0.05$ . B. Plasma (RA, n = 24; OA, n = 14), RA vs OA,  $p < 0.01$ . C. MNC lysate (RA or OA, n = 10), RA vs OA,  $p < 0.005$ . D. FLS lysate (RA or OA, n = 3), RA vs OA,  $p < 0.05$ .

levels of synovial fluid or plasma samples in patients with RA (synovial fluid,  $\gamma = 0.923$ ,  $p < 0.001$ ; plasma,  $\gamma = 0.650$ ,  $p < 0.001$ ).

## DISCUSSION

In our study both circulating and synovial levels of kallistatin were significantly increased in patients with RA. Since RA is characterized by the accumulation of activated synoviocytes and infiltrating MNC within the joint compartment, we further performed the immunohistochemical staining of kallistatin on synovial tissues to localize the cells responsible for its production<sup>11,12</sup>. Kallistatin was expressed on both infiltrating MNC and synoviocytes in rheumatoid synovia. Moreover, intracellular kallistatin levels were significantly elevated in MNC and FLS from patients with RA.

Inflammation, a hallmark of RA, is primarily driven by biological responses involving the formation of vasoactive peptides such as kinins<sup>2</sup>. The capacity of kinins to induce

release of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is critical in the maintenance of inflammation. TK has been described in synovial tissue extracts from patients with RA, and has been identified in both the synoviocytes and infiltrating polymorphonuclear leukocytes within rheumatoid joints<sup>3,13</sup>. TK activities were reported to be higher in synovial fluid from patients with RA as compared with that of patients with OA<sup>13</sup>. Kallistatin can inhibit TK activity *in vitro* by formation of a stable complex<sup>14</sup>. Coexpression and colocalization of kallistatin and TK in various tissues suggest an *in vivo* regulatory role of kallistatin on TK activities<sup>15</sup>. The kallikrein-kinin system is involved in inflammatory and autoimmune disorders<sup>2</sup>. In systemic sclerosis, higher circulating levels of TK were detected without changes in kallistatin levels, whereas in inflammatory bowel disease, intestinal concentrations of both TK and kallistatin were reduced<sup>16,17</sup>. Our studies revealed that higher circulating and synovial levels of kallistatin were significantly associated with increased TK concentrations. Increased

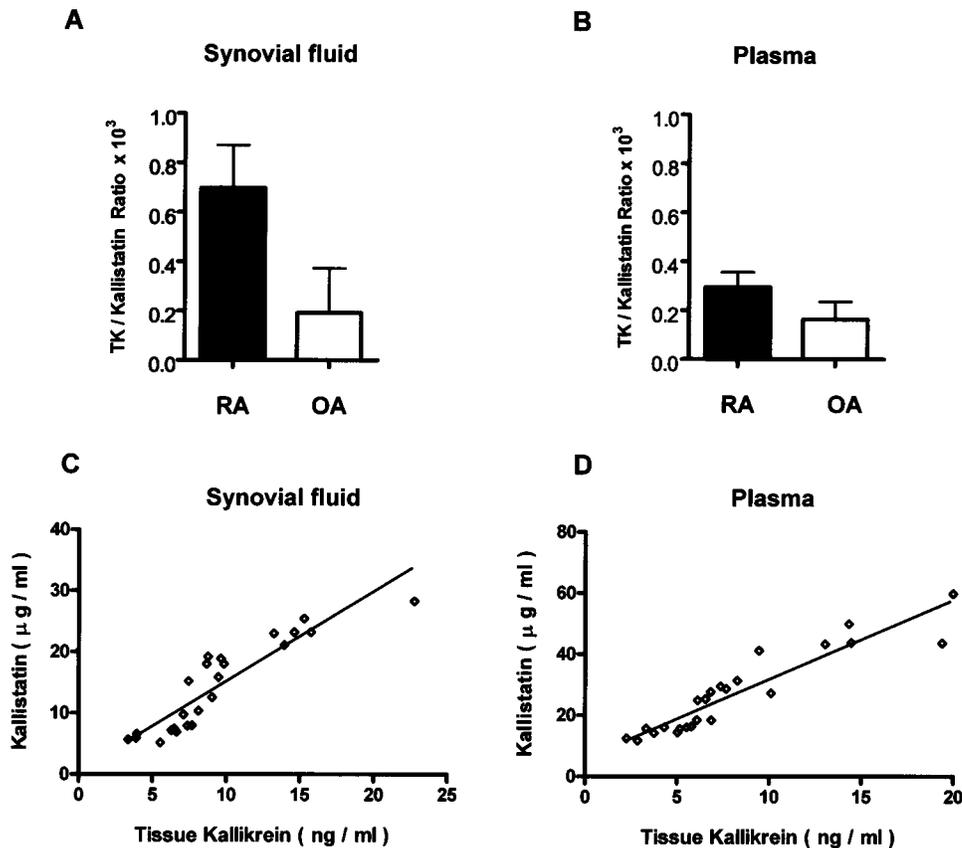


Figure 4. TK to kallistatin ratios of synovial fluid and plasma samples, and linear correlation figures between kallistatin and TK. A. Synovial fluid (RA, n = 24; OA, n = 14), RA vs OA,  $p < 0.001$ . B. Plasma (RA, n = 24; OA, n = 14), RA vs OA,  $p < 0.001$ . C. A significant linear correlation in synovial fluid from RA,  $\gamma = 0.964$ ,  $p < 0.001$ . D. A significant linear correlation in plasma from RA,  $\gamma = 0.960$ ,  $p < 0.001$ .

kallistatin production could exert a counter-regulation effect on increased angiogenesis induced by elevated TK levels in patients with RA. The TK to kallistatin ratios in synovial fluid and plasma samples were calculated in our study to measure how much the TK/kallistatin system is upregulated in patients with RA compared to those with OA. However, rather than similar values to those of patients with OA, the ratios were higher in patients with RA. Although the elevated levels of TK were strongly associated with increased concentrations of kallistatin, the amounts of kallistatin were not high enough in suppression of neovascularization within the rheumatoid joints. Therefore, in our previous study, administration of kallistatin through the intraarticular injection of adenovirus encoding the kallistatin gene clearly ameliorated the rat arthritis by inhibiting angiogenesis<sup>7</sup>.

Current RA therapies target the inflammatory consequences of autoimmune activation with the use of biological agents that inhibit TNF- $\alpha$ <sup>18</sup>. Results from large clinical trials highlight the effectiveness of anti-TNF- $\alpha$  agents in treating RA<sup>19</sup>. The kallistatin gene-treated rats had significantly lower intraarticular TNF- $\alpha$  concentrations<sup>7</sup>. Whether there is an

inhibitory effect by kallistatin on TNF- $\alpha$  levels within rheumatoid joints remains to be determined. Anti-TNF- $\alpha$  agents may block TNF- $\alpha$ -driven vascular endothelial growth factor (VEGF) angiogenesis, but would be ineffective where hypoxia is the main stimulus for VEGF upregulation<sup>20</sup>. Previous studies showed that kallistatin acts by competing with VEGF binding to heparin-sulfate proteoglycans and thus suppressing angiogenesis signaling cascades induced by VEGF<sup>20,21</sup>. Addition of antiangiogenesis agents such as kallistatin might prove to be beneficial in future optimizing therapy for RA refractory to TNF- $\alpha$  antagonist treatment.

Elevated kallistatin levels were observed in patients with RA, particularly in synovial tissues, FLS, and MNC. This report is the first to describe upregulation of kallistatin expression in rheumatoid joints.

#### ACKNOWLEDGMENT

We are indebted to Prof. Ming-Fei Liu, Department of Internal Medicine, College of Medicine, National Cheng Kung University, and Dr. Pei-Chih Chen, former Visiting Staff, Department of Internal Medicine, National Cheng Kung University Hospital, for providing clinical specimens.

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