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Original Research

Argatroban more effectively inhibits the thrombin activity in synovial fluid than naturally occurring thrombin inhibitors

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Abstract: The purpose of this study was to clarify the precise effect of argatroban on the inhibition of cytokine secretion induced by thrombin on synovial cells. The efficiency of thrombin inactivation by thrombin inhibitors was evaluated in human synovial fluids (SFs). In SFs from 13 osteoarthritis (OA) and 11 rheumatoid arthritis (RA) patients, thrombin, Factor Xa (FXa), plasmin activity, IL-6, MMP-3, VEGF, and D-dimer concentrations were measured. Tissue factor (TF) activity or IL-6, MMP-3, and VEGF secretion of human synovial cells with or without thrombin and argatroban were measured. The efficiency of thrombin inactivation in SFs was compared for thrombin inhibitors: argatroban, antithrombin III (ATIII), or heparin cofactor II (HCII). In SFs, thrombin, FXa, plasmin, D-dimer, IL-6, and MMP-3 were significantly higher in RA than in OA. In synovial cell experiments, TNF-alpha and thrombin enhanced TF activity on the cell surface, and IL-6, MMP-3, and VEGF secretion were enhanced by thrombin. Increased TF activity, and IL-6, MMP-3, and VEGF secretion induced by thrombin were inhibited by argatroban. In SFs, argatroban inactivated thrombin more effectively than ATIII or HCII. Since thrombin plays an important role in the disease activity of OA and RA, it is a potential therapeutic molecular target. Argatroban was the most effective anticoagulant to inhibit thrombin activity in SF. Intra-articular injection is ideal administration because it can deliver high dose of argatroban without high risk of systematic complication.

Key words: Thrombin, argatroban, antithrombin III, heparin cofactor II.

Introduction

Joint inflammation is one of the main pathological conditions in arthritis, and persistent synovial inflammation leads to cartilage and bone destruction. SF accumulation, one aspect of synovial inflammation, is a common symptom in OA (osteoarthritis) and RA (rheumatoid arthritis). In SF, degraded matrices and various chemical mediators such as interleukins, TNFs (tumor necrosis factors), MMPs (matrix metalloproteases), VEGFs (vascular endothelial growth factors), and ADAM-TS ("a disintegrin and metalloproteinases with thrombospondin motifs") promote synovial inflammation, neovascularization, and cartilage and bone degradation. The activation of coagulation factors, which are major serine proteases, are observed as complexes with inhibitors like thrombin antithrombin III complex (TAT) or cleaved prothrombin fragment 1+2 in SFs (1). Fibrin deposition is a common feature of inflamed synovium in OA (2). In RA, fibrin and collagen fragments within a joint are called rice bodies (1). Additionally, the presence of D-dimer in SFs, which is a degradative product of fibrin by fibrinolysis factors like plasmin and plasminogen activator, suggests that both the coagulation and fibrinolysis systems are activated in the joints of OA and RA (1). These phenomena are called extravascular coagulation or fibrinolysis (3).

Various substrates are cleaved by activated coagulation factors in SF. G-protein-coupled signaling molecules PAR (protease-activated receptor) -1, -2, -3, and -4 are activated by cleavage of their N-terminal peptides. In particular, PAR-1, -3, and 4 are known as thrombin receptors. Thrombin increases IL-6 and MMPs production and cell proliferation via PAR-1 in synovial cells (4-6). Yang et al. reported a relationship between the lack of PAR-1 and arthritis severity in terms of histological amelioration in synovial exudates, cartilage degradation, and bone damage using PAR-1-deficient mice (7). Additionally, thrombin cleaves fibronectin at Arg^{269}-Ala^{270} and generates a 29-KDa amino-terminal fibronectin fragment, which has a remarkable catabolic effect by increasing MMPs expression and suppressing proteoglycan synthesis in cartilage (8). Similarly, thrombin cleaves the Lys^{114}-Arg^{115} bond in syndecan-4 (9). Emillia et al. reported that thrombin induces proteoglycan release in cartilage explants (10). The concentration of thrombin-cleaved osteopontin in SF is associated with the disease severity of OA and RA (2, 11). These findings indicate that the generation of thrombin is involved in joint inflammation and degeneration. Therefore, the inhibition of thrombin activity may provide great benefits to joint homeostasis.

Argatroban is a small molecule arginine derivative that inhibits thrombin activity by reversibly binding to its active site (12). This synthetic anticoagulant has already proved useful as a clinical agent for thrombosis

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such as in chronic arterial occlusion and cerebral infarction. We previously reported the effectiveness of argatroban for disorders other than thrombosis, such as tumor cell metastasis (13, 14). In this study, we hypothesized that argatroban may suppress inflammation in joint. The purpose of this study was to clarify the precise effect of argatroban on the inhibition of TF activation and cytokine secretion by synovial cells. Furthermore, to elucidate the efficiency of thrombin inhibition by argatroban in SFs, thrombin inhibitors such as argatroban, ATIII and HCII was compared in human SFs because ATIII and HCII were naturally observed in SFs.

Materials and Methods

Synovial fluid

SF samples were collected in 3.8% sodium citrate (9 volumes of SF to 1 volume of anticoagulant) from 13 OA and 11 RA patients. RA patients were diagnosed based on the guidelines of the American Rheumatism Association. All patients provided informed consent. This study was approved by the Ethics Committee of Mie University.

Measurement of coagulation activity and cytokines in SFs

Thrombin, FXa, and plasmin activity were measured using synthetic substrates in TBS buffer (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20) (3093-v, 3094-v, 3104-v: Peptide Institute, Osaka, Japan) (15) (16). Thrombin (Mochida Pharmaceutical Co, Tokyo, Japan), FXa (Sigma-Aldrich, St Louis, MO, USA), and plasmin (Sigma-Aldrich) were used as standards. After a substrate was added into SF, MCA (7-amino-4-methylcoumarin) was measured using a spectrofluorometer. The excitation wavelength was set to 380 nm and emissions were monitored at 440 nm. IL-6, MMP-3, VEGF (R&D SYSTEMS, Minneapolis, MN, USA), and D-dimer (American Diagnostica, Stamford, CT, USA) concentrations in SFs were measured using an ELISA kit.

Thrombin inhibition assay in SFs

SFs were diluted 10 times by TBS buffer. Equal volumes of solutions of thrombin inhibitors argatroban, ATIII (Sigma-Aldrich), or HCII (Sigma-Aldrich) in TBS were added into diluted SFs with or without heparin. The final concentration of each inhibitor and heparin was 1 μM and 1 U/ml. After 30 min, thrombin activity was measured using a synthetic substrate (3093-v).

Human synovial cells

Human synovial cells were purchased from Cell Applications (San Diego, CA, USA). Synovial cells were cultured in synoviocyte growth medium following the manufacturer's instructions and used for *in vitro* applications.

Measurement of TF activity

TF activity was measured as factor X (FX) activation by the Factor VIIa (FVIIa)/TF complex in synovial cells (17-19). Synovial cells were starved in serum-free medium for 24 h (24-well plates, 2.0×10^5 cells/well). After starvation, cells were treated with IL-1 beta, TNF-alpha, or thrombin in the presence or absence of argatroban (Mitsubishi Tanabe Pharma, Osaka, Japan) for 4 h. Cells were washed twice with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.5) containing 5 mmol/L $CaCl_2$. Synovial cells were incubated in wells with 200 μL of HEPES-buffered saline containing 5 nM of FVIIa and 500 nM of FX for 1 h. The generated FXa was measured using 200 μM of synthetic substrate (3094-V) after incubation for 3 min at room temperature. Fluorescence intensity was determined by excitation at 394 nm followed by measuring fluorescence at 444 nm in a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Measurement of cytokines in culture medium

Synovial cells were starved in serum-free medium for 24 h (24-well plates, 2.0×10^5 cells/well). After starvation, cells were treated with thrombin in the presence or absence of argatroban for 24 h. IL-6, MMP-3, and VEGF concentrations in the medium were determined using an ELISA kit.

Statistical analysis

All *in vitro* experiments were performed in triplicate, and representative results are shown. Results were compared by ANOVA. Data were considered significant at $P<0.05$.

Results

Coagulation activity and cytokines in SFs

In SFs, activated coagulation and fibrinolysis factors such as thrombin, FXa, and plasmin were observed.

Table 1. Characteristics of OA and RA Patients. Levels of coagulation, fibrinolysis, and cytokines in SFs of OA and RA. Values given are means ± standard deviation. (*: Mann-Whitney test, #: X^2-test).

	OA	RA	P-value
Gender, male/female,n	**1/12**	**1/10**	**#0.90**
Age, mean (range)	73.6 (46-86)	63.6 (43-82)	*0.036
CPR (mg/dl)	NA	6.08 ± 2.01	NA
RF (U/ml)	NA	265.8 ± 360.7	NA
Kellgren / Lawrence grade, n	I:0, II:1, III:8, IV:4	NA	NA
Xa (ng/ml)	82.5 ± 42.1	313.9 ± 164.3	*0.0001
Thrombin (mU/ml)	74.1 ± 23.8	254.8 ± 82.5	*0.0037
D-dimer (μg/ml)	21.3 ± 13.2	237.7 ± 142.5	*0.0002
Plasmin (μg/ml)	1.49 ± 0.36	2.58 ± 0.89	*0.0045
IL-6 (pg/ml)	239.4 ± 375.6	952.4 ± 252.5	*0.0011
VEGF (ng/ml)	1.41 ± 0.59	3.1 ± 2.56	*0.0929
MMP-3 (μg/ml)	1.39±0.89	3.98±1.48	*0.0005

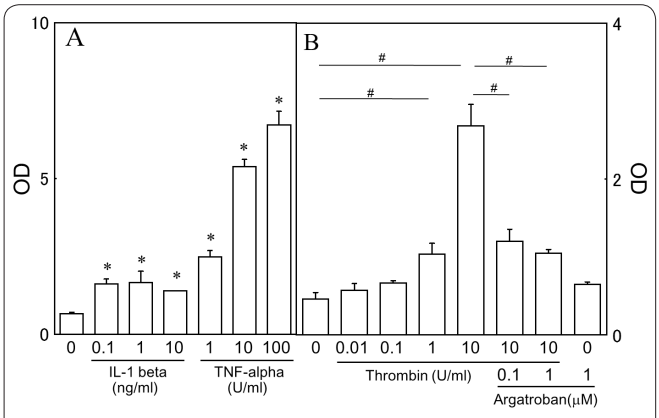

Figure 1. TF Activation by IL-1 beta, TNF-alpha, or Thrombin. After treatment with IL-1 beta, TNF-alpha, or thrombin in the presence or absence of argatroban, TF activity on synovial cells was measured. TNF-alpha and thrombin were potent inducers of TF activation (A, B). TF activation induced by thrombin was suppressed by argatroban dose-dependently (B). (* P<0.01 vs. control, # P<0.001).

While FXa and thrombin levels on average in RA were 2- to 4-fold higher than in OA, D-dimer levels were 10-fold higher (Table 1). A high concentration of D-dimer indicates the presence of high coagulation and fibrinolytic activity, with D-dimer being produced by proteolysis of crosslinked fibrin through plasmin. Additionally, the levels of IL-6 and MMP-3 were significantly higher in RA than OA, but VEGF was not (Table 1). A large amount of these coagulation and fibrinolysis factors and cytokines, especially in RA, exert a significant influence on joint homeostasis.

TF activity in synovial cells

In this study, TNF-alpha was an intensive inducer of TF in synovial cells (Figure 1A). This result supports the notion that SF in RA has high TNF-alpha concentrations and high coagulation activity (Figure 5, A) (1, 20). Additionally, we demonstrated that thrombin enhanced the TF activity of synovial cells in a dose-dependent manner (Figure 1B). This pathway is a positive feedback mechanism that plays an important role in forming the thrombogenic cycle (Figure 5, B). Additionally, argatroban inhibited TF activity induced by thrombin (-60.9% at 1 μM of argatroban vs. 10 U/ml of thrombin) (Figure 1B). This indicated that argatroban has the potential to interfere with the positive feedback pathway of thrombin to TF in synovial cells.

Thrombin induced cytokine secretions

In this study, the secretion of IL-6, MMP-3, and VEGF from synovial cells was significantly enhanced by thrombin in a dose-dependent manner (4.6-, 2.8-, and 2.4-fold higher at 10 U/ml of thrombin compared to controls, respectively) (Figure 2). Furthermore, argatroban inhibited the secretion of IL-6, MMP-3 and VEGF (-58.5%, -22.8% and -46.6% at 1 μM of argatroban vs. 10 U/ml of thrombin, respectively) induced by thrombin (Figure 2). From this data, thrombin plays an important role in disease activity and seems to be a therapeutic target. Argatroban has the potential to be a representative therapeutic agent for thrombin inhibition in SF.

Effect of thrombin inhibitors on thrombin activity in SFs

The effect of thrombin inhibitors was examined using three representative samples randomly chosen from OA or RA SFs, respectively. Thrombin activity in SFs was significantly reduced by Hepa+ATIII (OA: -28.1%, RA: -21.2% vs. control), and Hepa+HCII (OA: -18.1%, RA: -18.5% vs. control) in OA and RA SFs. Remarkably, argatroban inhibited thrombin activity much more than the natural anticoagulants (OA: -95.4%, RA: -72.3% vs. control) (Figure 3). Argatroban was the most effective agent for inhibiting thrombin in SFs.

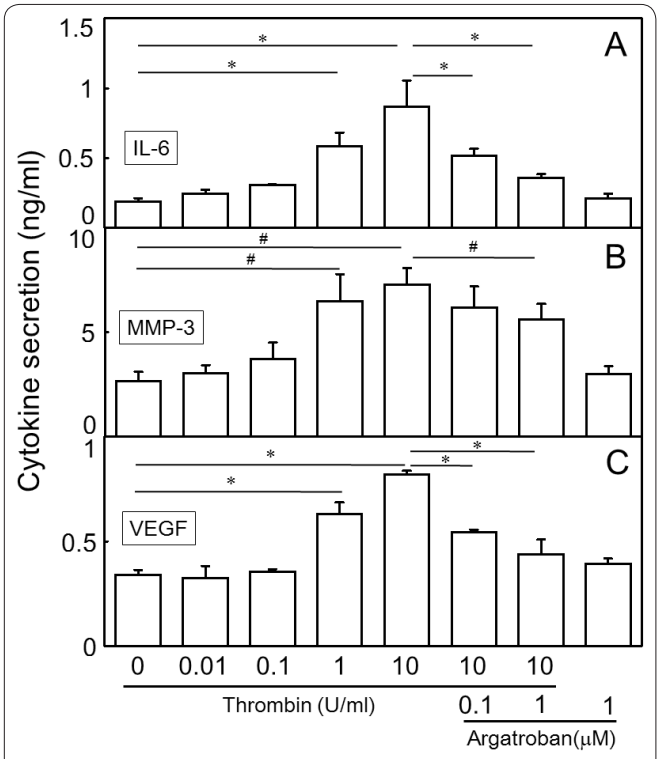

Figure 2. Cytokine Secretion by Thrombin. Thrombin (0.01-10U/ml) induced IL-6, MMP-3, and VEGF secretion from synovial cells in a dose-dependent manner. Induced IL-6, MMP-3, and VEGF secretion by thrombin was suppressed by argatroban (0.1-1μM) in a dose-dependent manner. (* P<0.0001, # P<0.05).

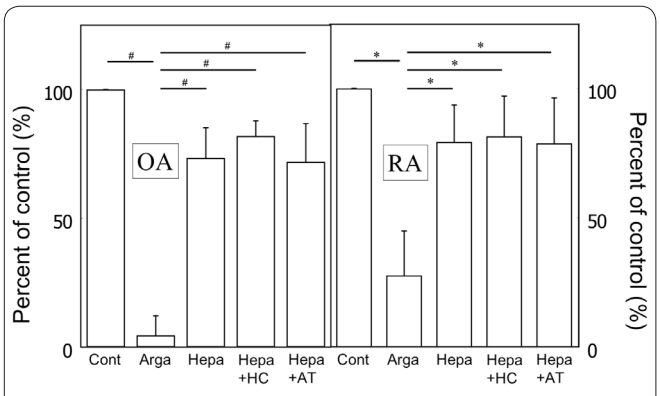

Figure 3. Thrombin Inactivation by Thrombin Inhibitors in SFs. Three representative SFs of OA (A) or RA (B) were examined. The average of three measurements for OA or RA are shown. Argatroban inhibited thrombin activity more than the other thrombin inhibitors. (Arga: 1μM of argatroban, Hepa: 1 U/ml, HCII: 1μM of Heparin cofactor II, AT: 1μM of antithrombin III) (# P<0.0001, * P<0.05).

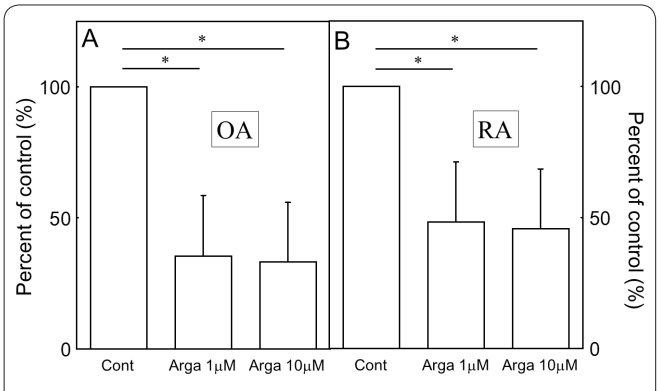

Figure 4. Dosage Effect on Thrombin Activity Inhibition by Argatroban in SFs. SFs from 13 OA patients or 11 RA patients were examined. 1 and 10 μM of argatroban significantly inhibited thrombin activity in OA or RA SFs. (* P<0.0001).

Dosage effect on thrombin activity inhibition by argatroban in SFs

All OA and RA SFs were used in the thrombin inhibition assay with argatroban. Since 0.1 μM of argatroban showed mild inhibition of thrombin in the *in vitro* study (Figure 1, 2), 1 and 10 μM of argatroban were examined in this assay. Both doses significantly reduced thrombin activity in SFs of OA (-65.6% at 1 μM, -66.8% at 10 μM vs. control) and RA (-51.7% at 1 μM, -54.4% at 10 μM vs. control). There was no significant difference between 1 and 10 μM of argatroban (Figure 4). From these data, we concluded that argatroban is an attractive choice for effective inactivation of thrombin in SF.

Discussion

The beginning of the coagulation cascade in the articular joint is thought to start with TF activation of synovial cells, B cells, and macrophages (21). Once TF is exposed to the extracellular environment, the extrinsic coagulation pathway cascade proceeds. After TF forms a complex with FVIIa, it converts FX into FXa. FXa activates prothrombin to thrombin and finally leads to fibrin production. In SFs, inflammatory effects by cytokines and coagulation factors is higher in RA than in OA (1). An intimate relationship between TNF-alpha and coagulation factors in SFs is demonstrated by the fact that TF activation was induced by TNF-alpha in synovial cells in the current study (Figure 1A). In this manner, inflammatory cytokines may occupy an important place in the regulation of the coagulation cascade by inducing TF activation in joint.

Furthermore, fibrin provides an environment for plasmin production due to the presence of fibrin binding sites in plasminogen and tissue-type plasminogen activator (t-PA) (22). Plasmin converted from plasminogen with t-PA leads to fibrin degradation and proteolysis. Plasmin cleaves aggrecan at the Arg^{394}-Gly^{395} bond in interglobular domain (23) and syndecan-4 at the Lys^{114}-Arg^{115} and Lys^{129}-Val^{130} bonds. It is well known that plasmin activates pro-MMPs including MMP-3 (24). These destructive effects by plasmin were partially generated by thrombin via fibrin formation. Surprisingly, while normal level of D-dimer in blood is known as 0.4-1μg/ml, D-dimer in SF of this study was extremely higher (average, OA: 21.3μg/ml, RA: 237.7μg/ml, Table 1). This indicated that intensive coagulation and fibrinolysis reaction proceeded in SFs.

Given these findings, the regulation of thrombin activity in SFs may be of great potential value for further clinical improvement. Hirudin, a natural thrombin inhibitor of the leech, reduces joint inflammation associated with antigen- or collagen-induced arthritis models (25, 26). Not only have TNF-alpha blockade agents been proved to be of enormous clinical benefit in RA therapy, but they ameliorate the plasma levels of D-dimer and prothrombin fragment 1+2 (27). This improvement of coagulation status by TNF-alpha inhibition leads us to expect that the coagulation activity in SFs will be reduced by cytokine blockade therapy. However, cytokine blockade therapy may be insufficient to inhibit thrombin generation in SFs without anticoagulation agents, because there are two cytokine-independent pathways that produce thrombin, one involving TF-bearing microparticles (MPs) in SFs (Figure 5, C), the other involving TF re-activation by thrombin (Figure 5, B). MPs are released from cells upon activation or during apoptosis. In particular, as MPs derived from monocytes and granulocytes in SFs have procoagulant activity by TF on the membrane, the activation of extrinsic pathway by MPs is not regulated by cytokines such as TNF-alpha (28). Secondly, we demonstrated that thrombin induced the re-expression of TF on synovial cells (Figure 1B). This pathway is a positive feedback mechanism that plays an important role in forming the thrombogenic cycle, as well as in smooth muscle cells and tumor cells (Figure 5, black arrows: TF→FXa→Thrombin→TF) (14, 29). In addition, citrullination is observed in coagulation factors in RA. The level of citrullinated ATIII is higher in RA plasma than in controls with non-arthritis disease and in healthy individuals. The loss of thrombin inhi-

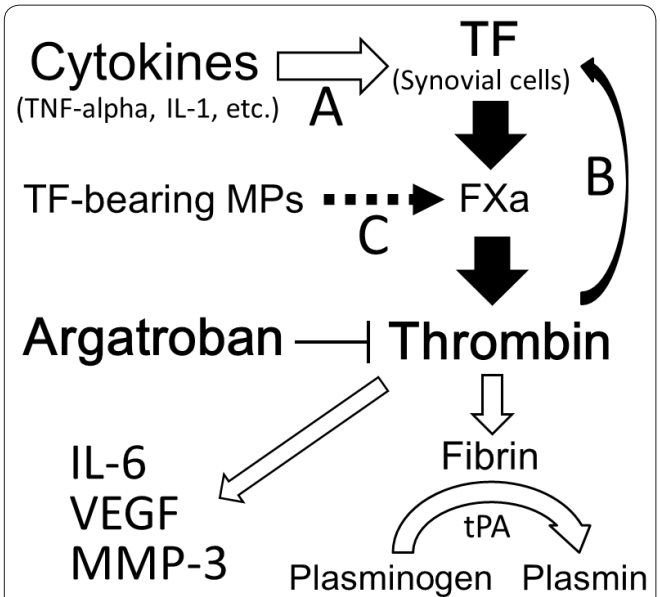

Figure 5. Thrombogenic Cycle. In this study, we demonstrated a pathway for TF activation on the synovial cells by thrombin (B). This creates a thrombogenic cycle on synovial cells (black arrows, TF→FXa→Thrombin→TF). A is a common and main pathway to thrombin generation by cytokine stimulation such as TNF alpha. B and C are cytokine-independent pathways to thrombin generation. Thrombin is a key factor of TF re- activation, IL-6, VEGF and MMP-3 secretion, and plasmin production. Thrombin inhibition beneficially interferes with the thrombogenic cycle, IL-6, VEGF and MMP-3 secretion, and plasmin production.

bitory activity of ATIII due to citrullination may lead to further thrombin generation and result in excessive fibrin deposition and inflammation (30, 31). Inactivation of thrombin is of great importance not only for the down-regulation of IL-6, MMP, and VEGF, but also to block further generation of thrombin from cytokine-dependent and -independent pathways such as MPs and the thrombogenic cycle (Figure 5).

ATIII and HCII, major natural thrombin inhibitors, did not effectively inhibit thrombin activity in SFs in this study. Conversely, argatroban strongly inhibited thrombin activity (Figure 4). Two possible reasons of this different result were speculated. Firstly, as argatroban does not need cofactors, it can inhibit thrombin directly. On the other hand, to obtain strong effect on thrombin inhibition, ATIII and HCII need to bind to cofactors like heparin. Secondly, it is thought to result from the difference of molecular weight. From Stokes-Einstein equation, diffusive coefficient (D) is decided by viscosity and molecular size (radius of the molecule). Lager molecular size or higher viscosity gives low D value. In the situation of low D value, molecular diffusion need more displacement time. In viscous fluids like SF, small molecules like argatroban (MW: 508.6) may be able to move and bind to thrombin more easily and smoothly than large molecules like ATIII (MW: 65,000) or HCII (MW: 66,000).

The conservative treatment for OA has not progressed in over 20 years. Hyaluronic acid and steroids are still the major drugs injected into articular joints. Argatroban is an attractive candidate to treat synovial inflammation. Systemic administration by venous infusion, common administration of argatroban, has a risk of bleeding and the infiltration from vascular into SF is minute. Intra-articular injection is ideal administration for treatment because it can deliver high dose of argatroban without high risk of systematic complication. Additionally, in intra-articular space, which is extravascular environment, argatroban will not prolong blood coagulation without vascular infiltration. Argatroban may have the potential to treat synovial inflammation and provide further clinical benefits. This study needs further study to clarify in vivo effects.

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