Inhibition of the insulin-like growth factor system is a potential therapy for rheumatoid arthritis

Satoshi Suzuki*, Shinji Morimoto#, Maki Fujishiro#, Mikiko Kawasaki#, Kunihiro Hayakawa#, Tomoko Miyashita#, Keigo Ikeda#*, Mitsuaki Yanagida#, Kenji Takamori#, Hideoki Ogawa#, Iwao Sekigawa#*, Yoshinari Takasaki#

#Institute for Environment and Gender Specific Medicine, Juntendo University Graduate School of Medicine, Chiba, 2-1-1 Tomioka, Urayasu, Chiba 279-0021, Japan.
*Department of Rheumatology, School of Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo, Tokyo 113-8421, Japan.
¶Department of Internal Medicine and Rheumatology, Juntendo University Urayasu Hospital, 2-1-1 Tomioka, Urayasu, Chiba 279-0021, Japan.

Corresponding author: Shinji Morimoto, MD, PhD

¶Department of Internal Medicine and Rheumatology, Juntendo University Urayasu Hospital, 2-1-1 Tomioka, Urayasu, Chiba 279-0021, Japan.
Tel: 81-4-7353-3111 Fax: 81-4-7381-5054 Email: morimoto@juntendo.ac.jp

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Abstract
**Introduction** We have shown that connective tissue growth factor (CTGF) plays an important role in the pathogenesis of rheumatoid arthritis (RA). Insulin-like Growth Factor Binding Proteins (IGFBPs) are one of module of CTGF and IGFBPs are a family of circulating proteins which bind Insulin-like Growth Factors-I and II (IGF-I and IGF-II). IGF-I promotes the growth and differentiation of the bone and cartilage tissue and also plays a role in the regulation of immunity and inflammation. Therefore, this study was focused on IGF system to investigate how IGF system is associated with the disease progression of RA.

**Methods** Serum samples were collected from RA patients in active or inactive disease stages. IGF-I and IGFBP3 production was evaluated by ELISA, RTPCR, indirect immunofluorescence microscopy. Osteoclastogenesis was evaluated using tartrate-resistant acid phosphatase (TRAP) staining, a bone resorption assay and osteoclasts specific catalytic enzymes productions. Angiogenesis was perfomed tube formation assay using human umbilical vein endothelial cells (HUVEC).

**Results** The serum concentrations of IGFBP3 in RA were greater than in normal healthy controls. Furthermore, tumour necrosis factor (TNF)α can induce the IGF-I and IGFBP3 production from synovial fibroblasts. IGF-I and IGFBP3 promoted the induction of the quantitative and qualitative activities of osteoclasts in combination with
M-CSF and receptor activator of NF-κB ligand (RANKL). In addition, IGF-I and IGFBP3 induced angiogenesis by tube formation assay using HUVEC. These effects was neutralized by adding anti-IGF-IR mAb.

**Conclusions** These results indicate that aberrant IGF-I and IGFBP3 production induced by TNFα plays a central role for the abnormal osteoclastic activation and angiogenesis in RA patients. The blockade of the IGF system might become a new useful strategy option for the treatment of RA.

**Introduction**
We previously reported that the contribution of connective tissue growth factor (CTGF) to the pathogenesis of RA and found that the serum levels and tissue expression of CTGF was upregulated in patients with RA (1). To extend our research of CTGF on the pathogenesis of RA, we found that blockade of CTGF pathway by administration of anti-CTGF monoclonal antibody (mAb) significantly prevented the progression of arthritis in CIA mice (2). CTGF is a member of the CCN protein family, whose members is characterized by significant sequence homology and show conservation of all 38 cysteine residues. These proteins are characterized by four homologous modules, Insulin-like Growth Factor Binding Proteins (IGFBP), von Willebrand factor type C repeat, thrombospondin type 1 repeat and C-terminal (CT) module (3).

Human IGFBPs, one of module of CTGF, are a family of circulating proteins which bind Insulin-like Growth Factors-I and II (IGF-I and IGF-II). IGFs are growth factors that have both metabolic and mitogenic activities and activate a tyrosine kinase pathway via binding to IGF-IR and IGF-IIR on cell surfaces. IGF-I promotes the growth and differentiation of the bone and cartilage tissue and also plays a role in the regulation of immunity and inflammation(4). For instance, proinflammatory mediators such as tumor necrosis factor a (TNF-α) and prostaglandin E₂ have been shown to induce IGF-I production in macrophages ((5)). In addition to their
IGF-dependent actions, the IGFBPs also exert IGF independent biological effects that involve neither binding of IGFs nor modulation of the IGF receptor\(^4\). IGFBPs regulate the bioavailability of IGF-I. Only free IGF-I is biologically active but nearly all IGF-I is bound to IGFBP-3, the most abundant binding protein in serum\(^6\) (7).

IGF-I exerts its action by binding to the IGF-I receptor (IGF-IR), which induces receptor autophosphorylation in the intracellular kinase domain. On receptor activation several protein substrates, including insulin receptor substrate 1 (IRS-1) and Src homolog and collagen protein (SHC), are activated, and transduce multiple signaling pathways, including the PI3K/PDK-1/Akt pathway and Ras/Raf-1/MAPK (mitogen-activated protein kinase) pathway. IRS-1 interacts with phosphatidylinositol-3-kinase (PI3K), and activation of PI3K catalyzes a phosphorylation of phosphatidylinositol-4,5-diphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). The increased PIP3 level activates phosphoinositide-dependent kinase 1 (PDK-1) and Akt. The Ras/Raf-1/MAPK pathway is activated through the SHC-Grb2-SOC complex, which is critical for cell proliferation (4).

Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic
inflammation of synovial joints, eventually resulting in the destruction of cartilage and bone (8). During RA, the synovial tissue becomes infiltrated by inflammatory cells and increases greatly in mass due to the tumor-like proliferation of activated synoviocytes. In response to the growing metabolic demand caused by synoviocyte proliferation, blood vessels develop which nourish and oxygenate this synovial pannus, promoting it to invade and degrade adjacent cartilage and bone. Neovessels exacerbate inflammation by further facilitating the ingress of inflammatory cells and mediators into the joint (9, 10). Targeting the synovial vasculature and bone destruction has therefore been proposed as a possible therapeutic strategy in RA.

Therefore, based on these findings, the contribution of IGF system, especially IGF-I/IGBP3 complex, for RA pathogenesis was investigated in the current study. Here, we report that aberrant IGF-I/IGBP3 complex production mediated by TNF-α can induce massive osteoclastogenesis and disturbance on homeostasis of cartilage resulting in bone and cartilage tissue damage in RA. The blockade of the IGF system might become a new useful strategy option for the treatment of RA.

Materials and methods
Patients and samples

All patients with RA fulfilled the American College of Rheumatology (ACR) criteria (11). Serum samples were obtained from 31 patients with RA and 20 normal age- and gender-nearly matched healthy volunteers. The synovial tissue samples were obtained from two patients with RA and osteoarthropathy (OA) as disease controls during a surgical operation for knee joints arthropathy. The patients with RA were further categorized as an active RA group (n = 7) and inactive RA group (n = 24) depending on the elevated serum C-reactive protein (CRP) level (normal range < 0.3 mg/dl). The precise clinical profiles of these patients have described in a Table 1. All patients provided their informed consent to participate in this study and the study was approved by the local ethics committee.

Materials and cell lines

A human synovial fibroblasts cell line, MH7A (Riken Cell Bank, Ibaraki, Japan), isolated from the knee joint of RA, was provided by Dr. Miyazawa (10). MH7A cells were stimulated with or without recombinant TNF-α (20 ng/ml in MH7A cells) (R&D System; Minneapolis, MN, USA) in appropriate time (6 and 24 or 48 hours) and used for the subsequent experiments. Treatment with 1 μg/ml of infliximab was used to
inhibit effects of TNF-α in vitro in the experiments.

**ELISA for human IGF-1 and IGFBP3**

The serum level of IGF-I and IGFBP3 in human sera was evaluated by a sandwich ELISA system using human IGF-I ELISA kit (Enzo Life Sciences, N.Y., USA) and human IGFBP-3 ELISA kit (Abnova corporation, Taipei, Taiwan) according to manufacturer’s instruction. Each sample was analyzed in triplicate and the average optical density (OD) at 460 nm with an appropriate development time was used for the data analysis.

**Total RNA extraction and real-time RT-PCR**

Total RNA was extracted from the MH7A cells and osteclasts using the Rneasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Strands of cDNA were synthesized using a PrimeScript RT reagent kit (Takara, Shiga, Japan) with 0.5 μg total RNA. Real-time RT-PCR using SYBR Premix Ex Taq Perfect real time (Takara) was used for the quantitation of CTGF mRNA. The primers for human IGF-I (GenBank:NP_000618.3) were designated as 5’ - TTTCAAGCCACCCATTGACC - 3’ (forward) and 3’ -
GCGGGTACAAGATAAATATCCAAAC - 5’ (reverse), human IGFBP3 (GenBank: NP_001013398.1) 5’ - TCTAGGGCACTCTGGGAACCTATAA - 3’ (forward) and 3’ - TTGTGATGCCTCTGAATGTGGA-5’ (reverse), β-actin primers for control primers as 5’-TGGCACCCAGCACAATGAA- 3’ (forward) and 5’-CTAAGTCATAGTCCGCCTAGAAGCA- 3’ (reverse). Quantitative real-time RTPCR was performed in 20 μl volume with 500 ng cDNA in SYBR Premix Ex Taq Kit (Takara). The amplification cycles consisted of 95°C for five seconds as first steps (one cycle), 95°C for five seconds and 60°C for 30 seconds for IGF-I and IGFBP3 as second steps (45 cycles), 95°C for five seconds and 60°C for 30 seconds and 95°C for 15 seconds as third steps (one cycle) according to protocol described in the manufacturer's instructions (Takara). To determine the quantitative expression levels of the transcripts, samples loading was monitored and normalized by the expression of β-actin transcripts.

**Osteoclasts differentiation**

Peripheral blood monocytes (PBMC) from healthy donors were collected using Ficoll-gradient centrifugation (Ficoll- Paque PLUS, GE Healthcare, Chalfont St Giles UK). The PBMC were purified into a CD14+ population using anti- CD14 MACS
microbeads (Miletenyi Biotec, Auburn, CA, USA) according to the protocol supplied by the manufacturer. A flow cytometry analysis using phycoerythrin (PE)-conjugated mouse anti-CD14 mAb (Miletenyi Biotec) showed that purity of the CD14+ monocytes was more than 98% in each experiment. The purified CD14+ monocytes (5 × 10^4 cells/well) were cultured in 96 wells in alpha minimum essential medium (αMEM, Invitrogen, Grand Island, NY, USA) with 10% FBS and incubated with M-CSF (25 ng/ml) and soluble RANKL (sRANKL; 40 ng/ml) (Millipore, Billerica, MA, USA) with or without recombinant human IGF-I (15ng/ml, R&D Systems), recombinant human IGFBP3 (15ng/ml, R&D Systems) and anti- human IGF-IR mAb (6 ng/ml, R&D Systems). The medium was replaced with fresh medium three days later and the cells were stained for tartrate-resistant acid phosphatase (TRAP) expression using a commercial kit (Cell Garage, Tokyo, Japan) after incubation for seven days. The number of TRAP positive multinucleated cells (MNC) in three randomly selected fields examined at 100× magnification of the total number of TRAP-positive MNC per well were counted as osteoclasts under light microscopy.

Immunohistochemistry analysis

A histochemical analysis with indirect immunofluorescence microscopy was performed.
Briefly, serial paraffin sections derived from surgical samples were deparaffinized, rehydrated and washed with PBS as previously reported (12). Triple staining for IGF-I, IGFBP3, and F4/80, which is widely used as a specific marker for macrophage, was performed. The samples were incubated with 10% bovine serum albumin (Sigma) for 60 minutes to eliminate nonspecific binding, and then incubated with rabbit anti-human IGF-I antibody (4 µg/ml, Abcam) and mouse anti-human IGFBP3 antibody (25 µg/ml, R&D Systems) and rat anti-human F4/80 antibody (Abcam) for 60 minutes diluted 1:50 in PBS. After washing, the bound antibodies were labeled with Alexa468 (Invitrogen Corporation, Carlsbad, CA, USA) conjugated anti-goat IgG antibody (Invitrogen Corporation, Carlsbad, CA, USA) and Alexa594 conjugated anti-rat IgG antibody (Molecular Probe) for detection of fluorescence images. The sections were counterstained by the nuclear stain 40,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). The other sections were also stained with hematoxylin/eosin (HE).

**Tube Formation Assay**

Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza (Walkersville, MD, USA). The medium from the bullet kit EGM-2 Set (Lonza),
containing 0.1% R3-IGF-I, 0.1% VEGF, 0.4% hFGF-2, 0.1% hEGF, 0.04% hydrocortisone, 0.1% ascorbic acid, 0.1% heparin, 0.1% GA-100 and 2% FBS, was used for culture, and HUVEC from the 3rd passage were used in the analyses. Thirty microliters of phenol red-free Growth Factor Reduced Matrigel Matrix (BD, Franklin Lakes, NJ, USA) was placed in each well of 96-well plates, and subsequently recombinant human IGFBP3 (0.1 μg/ml, R&D Systems) and anti-human IGF-IR mAb (6 ng/ml, R&D Systems) were added to each well. After matrigel solidified sufficiently at 37°C, 50 µl of HUVEC culture (concentration adjusted to 4 × 10^5 cells/ml) was added to each well and the plates were incubated overnight. The number of junctions in 3 fields was counted for each well by using a light microscope, and the average of the values from the 3 fields were used for comparison.

**Statistical analysis**

The experimental data were compared using un-paired Student's t-test with P values < 0.05 considered to be statistically significant.

**Results**

*Increased serum levels of IGFBP3 in patients with RA*
Figure 1 shows the serum levels of IGF-I and IGFBP3 in the patients with non-active RA, active RA and normal controls. The serum levels of IGFBP3 in RA (inactive and active) patients (Figure 1B) were significantly greater in comparison to normal controls ($P < 0.05$). There were no significant differences in the serum IGF-I concentrations between RA and normal controls.

**Effects of TNF-α for the production of IGF-I and IGFBP3 from synovial fibroblasts**

To investigate the IGF-I/IGFBP3 complex contribution for pathogenesis of RA, the IGF-I and IGFBP3 expression was evaluated by immunohistochemical analysis in synovial tissues of surgical samples from the knee joint of RA patients and OA patients as a disease control. An inflamed synovial tissue was recognized in samples from RA compared to OA in HE staining (Figure 2A). A strong expression of IGF-I and IGFBP3 was observed in inflamed synovial tissue with RA, and none or very weak expression was recognized in samples with OA (Figure 2B, C). Inflamed synovial tissue of RA generally consists of fibroblasts and lineage of hemapoietic cells such as macrophage, neutrophils, and lymphocytes. To investigate a more specific production site of IGF-I and IGFBP3, the samples were also stained by anti-F4/80 antibody, which is generally
used for a specific marker of macrophages. As expected, a significant infiltration of macrophages was observed in RA compared to OA (Figure 2D). Triple staining using anti-IGF-I antibody, anti-IGFBP3 antibody and anti-F4/80 antibody showed expression of IGF-I, IGFBP3 and F4/80 was not overlapped (Figure 2E), suggesting that these molecules were produced at different sites. Because it has been reported that IGF-I was not generally expressed in hemopoietic lineage cells (4), we considered that IGF-I and IGFBP3 was mainly produced at synovial fibroblasts in inflamed synovial tissues with RA rather than hemopoietic cells like macrophages.

In order to investigate how TNF-α regulates the production of IGF-I and IGFBP3 in synovial fibroblasts, IGF-I and IGFBP3 expression was measured in MH7A cells (human synovial fibroblasts cell line) stimulated with or without recombinant TNF-α. The results obtained by quantitative real time PCR revealed that TNF-α enhanced the expression of IGF-I and IGFBP3 in MH7A cells and this enhancement was neutralized by anti-IGF1R mAb (Figure 3A). These data suggest that TNF-α can distinctly regulate IGF-I and IGFBP3 production in a cell dependent manner.

Synergistic effects of IGF-I and IGFBP3 on osteoclastgenesis mediated with M-CSF/RANKL was neutralized by anti-IGF1R mAb
Next, the effect of IGF-I and IGFBP3 on osteoclastogenesis was examined in order to investigate functional roles of IGF system on the RA related bone destruction. An expected, MCSF/ sRANKL-mediated osteoclastogenesis was enhanced by the presence of IGF-I and IGFBP3 not only in the morphological size but also in the number of osteoclasts (Figure 4C) and this enhancing effect was neutralized by anti-IGF-IR mAb (Figure 4D).

**Effect of IGF-I and IGFBP3 on Angiogenesis and inhibited by anti-IGF-IR mAb in In Vitro Assays**

One of the important steps during neo-angiogenesis is the formation and merging of tubes produced by endothelial cells forming a complex network of vessels and capillaries (13, 14). To understand the angiogenic activity of the IGF-I and IGFBP3 in *in vivo*, we performed tube formation assay. As shown in Figure 5B, IGF-I and/or IGFBP3 treatment induced the formation of tubular networks by HUVEC, which was disrupted by adding an anti-IGF-1R mAb Fig. 5C).

**Discussion**

This study investigated roles of IGF system for the possible pathogenesis and
therapeutic strategy of RA. We found novel findings as follows:  
a) serum levels of IGFBP3 in RA (inactive and active) were significantly greater than those of normal healthy controls (Figure 1B).  
b) Immunohistochemical studies reveal that IGF-I and IGFBP3 appears to be massively produced by synovial fibroblasts in RA (Figure 2).  
c) In rheumatoid arthritis synovial fibroblasts (RASF), TNFα upregulated the production of IGF-I and IGFBP3 mRNA. Moreover, these effects were neutralized by adding an anti-IGF-IR mAb (Figure 3).  
d) IGF-I and IGFBP3 possessed a synergistic effect in combination with MCSF/RANKL for osteoclastogenesis on the osteoclasts and this osteoclastogenesis was neutralized by adding an anti-IGF-IR mAb (Figures 4).  
e) On tube formation assay, IGF-I and/or IGFBP3 treatment induced the formation of tubular networks by HUVEC, it means an acceleration of angiogenesis that cause pannus at RA synoviocytes, which was disrupted by adding an anti-IGF-IR mAb (Figure 5).

Our data showed that no significant differences in the serum IGF-I concentrations between RA and healthy controls. However, immunohistochemical studies revealed that IGF-I and IGFBP3 appears to be massively produced by synovial fibroblasts in RA. This discrepancy might be explained by the fact that IGF-I in synovial fluid reflects the local metabolism of cartilage tissue and that IGF-I in serum reflects the systemic
metabolism of tissue including bone and cartilage. Supporting our data, previous report showed that IGF-I levels were significantly higher in synovial fluid of patients with RA than those in OA (15).

We also investigated TNFα upregulated the production of IGF-I and IGFBP3 mRNA in RASF. Previous report showed that tumor necrosis factor a (TNF-α) and prostaglandin E2 have been shown to induce IGF-I production in macrophages (5). Strikingly, TNF-α enhanced the expression of IGF-I and IGFBP3 in MH7A cells and this enhancement was neutralized by adding anti-IGF-IR mAb (Figure 3A). These data suggest that TNF-α can distinctly regulate IGF-I and IGFBP3 production in a cell dependent manner. Moreover, we indicated IGF-I and/or IGFBP3 possessed a synergistic effect in combination with MCSF/RANKL for osteoclastogenesis on the osteoclasts. In view of instruction osteoclastgenesis, IGF-I induced activation of osteoclasts, either direct via IGF-receptor stimulation or indirect via RANKL-synthesis by osteoblasts, and therefore contributes to matrix degradation (16-18). And, IGFBPs regulate the bioavailability of IGF-I. Only free IGF-I is biologically active but nearly all IGF-I is bound to IGFBP-3, the most abundant binding protein in serum. Thus, the IGF-I/IGFBP-3 ratio may serve as a mass for the free and bioactive IGF-I (6, 7). These
are suggested that IGF-I and IGFBP3 possessed a synergistic effect in combination with MCSF/RANKL for osteoclastogenesis on the osteoclasts in RA.

On the other hand, we showed IGF-I, IGFBP3 treatment induced the formation of tubular networks by HUVEC, it means an acceleration of angiogenesis of RA synoviocytes. In RA, a luxuriant vasculature is an early feature of the arthritic synovium, and the number of synovial blood vessels correlates with hyperplasia, mononuclear cell infiltration and indices of joint tenderness (19). The vascular turnover in the arthritic synovium is increased, and synovial endothelial cells express markers of proliferation (20). Although the hyperplastic RA synovium is highly vascularised, paradoxically the tissue environment is chronically hypoxic (21). Synovial fluids from RA joints have been shown to promote endothelial cell migration and proliferation, and to induce vessel formation in an angiogenesis assay (22, 23), which reflects an active, proangiogenic phenotype of the arthritic synovium.

The IGF-IR comprises two extracellular α-subunits and two transmembrane β-subunits which are linked by disulfide bonds(24). Ligand binding to the α-subunits leads to activation of the intrinsic tyrosine kinases of the β-subunits, One principal
pathway that emerges after tyrosine phosphorylation of the IGF-I receptor involves the activation of PI3K. This leads to the recruitment of Akt (protein kinase B) and its subsequent activation by phosphoinositide dependent kinase-1 and -2. A second well-known pathway concerns activation of extracellular-regulated kinase (ERK)-1 and -2, which are isoforms of the mitogen-activated protein kinase (MAPK) family. It is now becoming clear that the IGF-IR also signals via activation of the JAK/STAT pathway (25). This pathway is activated by cytokines and hormones signaling through members of the hematopoietic/ cytokine receptor superfamily (26). IGF-1 stimulates the activation of STAT-1 and STAT-3 in several cell types (27-30). Although the mechanism of STAT phosphorylation and activation through the IGF-1R has not been fully elucidated yet, there are indications that JAKs are involved. For example, IGF-I stimulates the activation of JAK-1 and JAK-2 (31), and dominant negative mutants of either JAK-1 or -2 attenuate STAT-3 phosphorylation by IGF-1 (29). The finding that STAT-3 is associated to the IGF-1R and JAK-1 (27) is in line with this hypothesis. Activation of the JAK/STAT route by the IGF-1R may have important consequences for IGF-1 signaling. This pathway does not only enable the IGF-1R to regulate the activation of promoter regions containing consensus sites for STAT molecules, but also induces a feed-back mechanism independent of tyrosine phosphatases. Recently, PI3K
inhibitor has potential as a therapeutic agent for RA (32) and JAK inhibitor came to be
used as a therapeutic drug of RA (33). An anti-IGF-R mAb has ability to inhibit both
PI3K/PKT pathway and JAK/STAT pathway. This suggests that anti-IGF-IR mAb has
more powerful effect as a therapeutic agent of RA.

We showed here indicated an important role of IGF system in the development of
the bone destruction in patients with RA and suggested a mechanism explaining the
efficacy of anti- TNF-α antibodies in the prevention of bone destruction in RA. The
present data suggest that IGF system plays significant roles in the pathogenesis of RA
especially through aberrant activation of osteoclasts and disturbance of cartilage tissue
homeostasis, thus resulting in articular destruction. In addition, IGF system plays
significant roles in an acceleration of angiogenesis of RA synoviocytes. Taken together,
it is possible that the blockade of the IGF system signaling pathway by anti-IGF-IR
mAb has beneficial effects in the treatment of RA. Originally, anti-IGF-1R mAb has
been developed as a cancer therapeutic agent (34). Our data indicated that an
anti-IGF-IR mAb has potential as a therapeutic agent for RA in addition to its use as an
anti-tumor agent and anti-IGF-IR mAb has merit further investigation as promising
anti-rheumatic agent. The administration of anti-IGF-1R antibodies to RA model mice
in vivo will be conducted in the future. These results may open new therapeutic strategies for patients with RA and the possibility of development of more specific biological therapies rather than antibodies to TNF-α.

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Conflict of Interest: none
Figure Legends

Figure 1
The serum levels of IGF-I, IGFBP-3 in patients with rheumatoid arthritis. The serum concentration of insulin-like growth factor I (IGF-I) (Figure 1A) and IGF binding protein (IGFBP) 3 (Figure 1B) in patients with RA (n = 31) and normal healthy controls (n = 11) were measured using a sandwich ELISA. Bars in Figure 1A and 1B indicate the SD.

Figure 2
IGF-I and IGFBP3 expression in synovial tissue of patients with rheumatoid arthritis. The representative results of HE staining (Figure 2A), immunofluorescence anti-IGFBP3 antibody staining (Figure 2B; green), anti-IGF-I antibody staining (Figure 2C; red) anti-F4/80 antibody staining (Figure 2D; blue) are shown using surgical samples from RA and OA patients. The samples were counterstained by DAPI (blue) for nuclear staining and merge images are shown (Figure 2E). A strong expression of IGFBP3, IGF-I and F4/80 was observed in the samples of RA compared to OA and the IGFBP3 and IGF-I expression cells were not overlapped with F/40 expression cells indicating
that IGFBP3 and IGF-I is upregulated in synovial fibroblasts rather than macrophages.

**Figure 3**

Effects of TNFα regulation of IGF-I and IGFBP3 production in articular cells. IGF-I and IGFBP3 production in the human synovial fibroblasts cell line (MH7A) (Figure 3A, B) stimulated with/without TNF-α were evaluated by quantitative real time PCR. TNF-α promoted IGF-I and IGFBP3 production in MH7A.

**Figure 4**

Synergistic effects of IGF-I and IGFBP3 for M-CSF/sRANKL-mediated osteoclastogenesis. Figure 4A shows pictures of TRAP staining and Figure 4B, C shows the numbers of TRAP positive cells. The TRAP positive cells stained by red were indicated by arrows. IGF-I plus IGFBP3 increased not only number but also morphological size of differentiated osteoclasts in combination with M-CSF/RANKL and this effect was neutralized by adding anti- IGF-IR monoclonal antibody (Figure 4D). Bars in Figure 4B indicate the SD.

**Figure 5**

Anti- IGF-IR monoclonal antibody inhibit angiogenesis in in vitro models. (A) anti-
IGF-IR mAb inhibit IGF-I and IGFBP3 induced tube formation in HUVEC. HUVEC were plated on matrigel and effect of diastereoisomers treatment on IGF-1/IGFBP3-induced tube formation was analyzed. Representative tubular network photomicrographs are shown at 100x (top panel). Tube junction was quantified as detailed in ‘Methods’ (bottom panel). Abbreviations: *, p ≤ 0.001.
References
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### Patient profile with active/inactive rheumatoid arthritis

<table>
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<tr>
<th></th>
<th>Active RA</th>
<th>Inactive RA</th>
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<td>Median age (y.o)</td>
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<td>103.1 ± 73.3</td>
<td>&lt;15.0</td>
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</table>
**Figure 1**

A

B

*Serum level of IGF-I (pg/ml)*

RA Active RA Normal

*Serum level of IGFBP-3 (pg/ml)*

RA Active RA Normal

*p < 0.01*
Figure 2A

IGF-1

F4/80

Dapi

Overlay

HE
Figure 2B

IGFBP-3

F4/80

Dapi

Overlay

HE
Figure 3

(A) Relative normalized expression (IGF-I/ACTB)

(B) Relative normalized expression (IGFBP-3/ACTB)

*P < 0.05

* p < 0.05
M-CSF alone

M-CSF+RANKL

M-CSF+RANKL+IGF-I+IGFBP-3

M-CSF+RANKL+IGF-I+IGFBP-3 +anti IGF-IR mAb

Figure 4A
A: M-CSF 25ng/ml
B: M-CSF 25ng/ml+RANKL 40ng/ml
C: B+IGF-I 15ng/ml+IGFBP-3 15ng/ml
D: C+anti-IGF-IR Ab 6ng/ml

Average of A-D, which was observed by each well3 view

*p < 0.001
Figure 5

A, control  

B, IGFBP3 0.1μg/ml

C, IGFBP3 0.1μg/ml + anti-IGF1R Ab 6ng/ml

D

P<0.01

count to junction

control  

IGFBP3 0.1μg/ml

IGFBP3 0.1μg/ml + anti-IGF1R Ab 6ng/ml