Metformin repositioning in rheumatoid arthritis

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Abstract

Objective

Metformin is a known therapeutic agent for diabetes. Recently, several reports suggested the possibility of improvement in autoimmune disease and malignancy conditions through the effect of metformin on the immune system. Although there have been reports on the therapeutic effects of metformin on mouse models of collagen-induced arthritis, simulating human rheumatoid arthritis (RA), the effect of metformin on human RA remains unknown. Therefore, we investigated the inhibitory effect of metformin on the pathogenesis of human RA in vitro.

Methods

Osteoclastogenesis was evaluated with or without metformin. through tartrate-resistant acid phosphatase staining, osteoclast-specific enzyme expression analysis, and a bone resorption assay. Human fibroblast-like synoviocyte MH7A cells were stimulated with TNF-α, and the expression of proinflammatory cytokines and protease and growth factor genes was evaluated with or without metformin. Metformin has been used to evaluate their potential modulatory effects on cells treated with TNF-α. Moreover, we examined angiogenesis by performing a tube formation assay using human umbilical vein endothelial cells (HUVECs) with or without metformin.

Results

Osteoclastogenesis was suppressed in the presence of metformin, and the expression of osteoclast-specific genes was reduced. The TNF-α-induced expression of inflammatory cytokines and protease and growth factor genes in MH7A cells was downregulated by metformin. Additionally, the induced formation of tubular networks in HUVECs was also disrupted following treatment with metformin.

Conclusion

These results suggest that metformin might improve the pathogenesis of RA, including joint inflammation and destruction. Thus, metformin might be utilised as a potential therapeutic agent in the treatment of RA.

Key words

rheumatoid arthritis, metformin, drug repositioning

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by osteoclastogenesis, synovial inflammation and proliferation, and pannus formation with aberrant angiogenesis resulting in the destruction of cartilage and bone. Osteoclasts, synovial fibroblast cells, and vascular endothelial cells are thought to be the main effector cells for the resulting bone destruction in RA (1). Metformin is an antidiabetic drug that lowers the levels of glucose by suppressing hepatic gluconeogenesis, reducing the intestinal transport of glucose, and increasing the peripheral uptake and utilisation of glucose. The mechanism of action of metformin at molecular level, metformin has been shown to act via both AMP-activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms; by inhibition of mitochondrial respiration but also perhaps by inhibition of mitochondrial glycerophosphate dehydrogenase, and a mechanism involving the lysosome (2). Although metformin is known to be derived from a natural product and not designed to target a particular pathway, however, its pharmacological effect is still not fully understood (2). Recent reports indicated that metformin exhibits pleiotropic action on other clinical domains, including cancer and cardiovascular disease (3). More recently, there have been reports suggesting that metformin could improve some autoimmune diseases (4). For example, in mice with collagen-induced arthritis (CIA) used to simulate the human rheumatoid arthritis (RA) model, metformin was shown to reduce their pathologies (5).

Therefore, on the basis of these findings, we investigated whether metformin was also effective in an *in vitro* RA model using human cells. No previous studies have examined the anti-inflammatory effects of metformin using human cells. We reported here that metformin suppressed osteoclastogenesis, and tumour necrosis α (TNF-α)-induced synovial cell inflammation and angiogenesis. Our study suggested that metformin has a potential for drug repositioning in RA.

Materials and methods

Cell lines

The MH7A cell line, which is synovial fibroblast cells isolated from the knee joint of patients with RA (6) was obtained from the Riken Cell Bank (Ibaraki, Japan). Human umbilical vein endothelial cells (HUVECs) were obtained from LONZA (Walkersville, MD, USA).

Reagents

Human recombinant TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). Metformin was purchased from Sigma-Aldrich Japan (Tokyo, Japan).

Osteoclast differentiation

Peripheral blood mononuclear cells (PBMCs) were obtained from the blood of healthy donors using Ficoll (GE Healthcare, Buckinghamshire, UK), while $CD14^+$ cells were purified as previously described (7). Flow cytometry analysis using phycoerythrin-conjugated antihuman anti-CD14 monoclonal antibody (Miletenyi Biotec, Bergisch Gladbach, Germany) showed that the purity of $CD14^+$ monocytes was more than 98% in each experiment. Purified $CD14^+$ monocytes were seeded in 96-well plates (5×10^4 cells/well) for tartrate-resistant acid phosphatase (TRAP) staining and in 24-well plates (2.5×10^5 cells/well) for quantitative real-time PCR. Cells were cultured in α-minimum essential medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and incubated with macrophage-colony stimulating factor (M-CSF) (25 ng/mL) (Merck Millipore, Darmstadt, Germany) and soluble receptor activator of NF-κB ligand (RANKL) (sRANKL; 40 ng/mL; Merck Millipore) with or without metformin (1 μM) for 7 days, and then cells were subjected to TRAP staining or quantitative real-time PCR analysis.

TRAP staining

TRAP staining was performed using the TRAP staining kit (Cosmo Bio, Tokyo, Japan) according to the manufacturer's instructions. The number of TRAP-positive-multinucleated cells (5 or more nuclei) were counted as osteoclasts, under light microscopy in 3 ran-

domly selected fields at 100× magnification, and the average of the obtained values was used for comparison.

Resorption assay

Purified $CD14^+$ monocytes were cultured on a Corning Osteo Assay Surface microplate (Corning Life Sciences, Tewksbury, MA, USA) with sRANKL (40 ng/mL) and M-CSF (25 ng/mL) in combination with or without metformin (1 μM) for 7 days, and then cells were lysed with the bleach solution (6% NaOCl, 5.2% NaCl). The area of resorption lacunae was measured in 3 randomly selected fields at 100× magnification under light microscopy, and the average of the recorded values was used for comparison.

MH7A cell inhibition assay

MH7A cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL)and grown in a standard T75 cell culture flask (Corning, Corning, NY, USA). Cells were maintained at 37°C and 5% CO_2 and grown until ~80–90% confluent. For new passages, between 1×10^6 cells were seeded into T75 flasks and grown to 80–90% confluence. The cells were removed by adding Trypsin-EDTA (Corning, Manassas, VA, USA) and the cell number was counted using a Millipore Sceptor Sensor (Millipore, Billerica, MA, USA). At passages 4, 9, 14, and 19, in addition to a T75 flask, a six-well plate was seeded by adding 1×10^6 cells per well. Once the cells reached confluence, all wells were rinsed with phosphate buffered saline (PBS; Sigma, St. Louis, MO, USA). MH7A cells were seeded on 6-well plates at a density of 1×10^6 cells per well. After incubating for 24 h in a CO_2 incubator (Thermo Fisher Scientific, Tokyo, Japan) at 37°C, semiconfluent cells were pretreated with or without metformin (1 mM) for 1 h and exposed to TNF-α (10 ng/mL) for 24 h. Then, cells were subjected to quantitative real-time PCR analysis.

Total RNA extraction and real-time RT-PCR

Real-time RT-PCR was performed as previously described (8). Briefly, total RNA was extracted from MH7A cells and osteoclasts, and reverse transcribed cDNA was obtained using the PrimeScript RT reagent kit (Takara, Shiga, Japan). Real-time PCR was performed using a SYBR Premix Ex Taq kit (Takara) in the ABI Prism 7900HT instrument (Applied Biosystems, Foster City, CA, USA). The expression of β-actin was used for normalisation. The following primer sequences were used: β-actin, 5′-TGGCACCCAGCACAATGAA-3′ (forward) and 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′ (reverse); cathepsin K (CTSK), 5′-AGCTGCAATAGCATAATCTGAACC-3′ (forward) and 5′-CGTTGTTCTTATTTCGAGCCATGA-3′ (reverse); matrix metallopeptidase 9 (MMP-9), 5′-ACCTCGAACTTTGACAGCGACA-3′ (forward) and 5′-GATGCCATTCACGTCGTCCTTA-3′ (reverse); interleukin 6 (IL-6), 5′- AAGCCAGAGCTGTGCAGATGAGTA-3′ (forward) and 5′-TGTCCTGCAGCCACTGGTTC-3′ (reverse); IL-1β, 5′- TGAAGCCCTTGCTGTAGTGGTG-3′ (forward) and 5′-GCTGATGGCCCTAAACAGATGAA-3′ (reverse); MMP-3, 5′-CTGGGCCAGGGATTAATGGAG-3′ (forward) and 5′-CAATTTCATGAGCAGCAACGAGA-3′ (reverse); insulin-like growth factor 1 (IGF-1), 5′-TTTCAAGCCACCCATTGACC-3′’ (forward) and 5′-GCG GGTACAAGATAAATATCCAAAC-3′ (reverse); and IGF-1 receptor (IGF-1R), 5′-ACCTCATTGGCCATGGAAACA-3′ (forward) and 5′-GACAATGACGGCAGCCAGAC-5′ (reverse). Relative expression levels were calculated using the delta delta Ct ($2^{-\Delta\Delta Ct}$; threshold cycle) comparative method.

Tube formation assay

HUVECs were cultured in an EGM-2 Bullet Kit set (EBM-2 medium containing 2% FBS and trophic factors; Lonza). Tube formation on Matrigel (Corning) was evaluated as a model of *in vitro* angiogenesis, and the assay was performed as previously described (9). Briefly, 30 μL of the growth factor-reduced Matrigel matrix was added into a 96-well plate with or without metformin (1 mM). After solidification, 50 μL of HUVEC cells (4×10^5 cells/mL, serum free) were seeded to each well and incubated at 37°C overnight. The number of junctions in 3 fields in each well was determined under a light microscope at 100× magnification, and the average number calculated from the 3 fields was used for comparison.

Statistical analysis

Data were expressed as mean ± SD for each sample. Statistical analyses were performed using the Student's two-tailed *t*-test for comparison of data in different groups. *p*-values of <0.05 were considered to indicate a statistically significant difference.

Results

Prevention of M-CSF/RANKL-induced human osteoclastogenesis by metformin

First, we investigated whether the addition of metformin resulted in suppression of osteoclastogenesis in $CD14^+$ monocytes treated with M-CSF and sRANKL with or without metformin. Accordingly, we found that TRAP positive osteoclasts were significantly decreased following treatment with metformin (Fig. 1A). Both the gene expression of cathepsin K, a gene related to bone destruction and MMP-9, an osteoclast marker, were demonstrated to be reduced when using metformin during M-CSF/RANKL-induced osteoclastogenesis (Fig. 1B). Additionally, the results of the bone resorption assay, used to show the function of osteoclasts, indicated a reduction of osteoclast-mediated representing vacant areas following treatment with metformin (Fig. 1C).

Suppression of TNF-α-induced inflammatory response in human synovial fibroblast cell line (MH7A) by metformin

We investigated whether addition of metformin resulted in the suppression of the TNF-α-induced expression of inflammatory cytokines and protease in MH7A cells. Respectively, we observed that the gene expression of the IL-6 and IL-1β inflammatory cytokines, and the expression of matrix metalloproteinases (MMP)-3, a protease related to joint

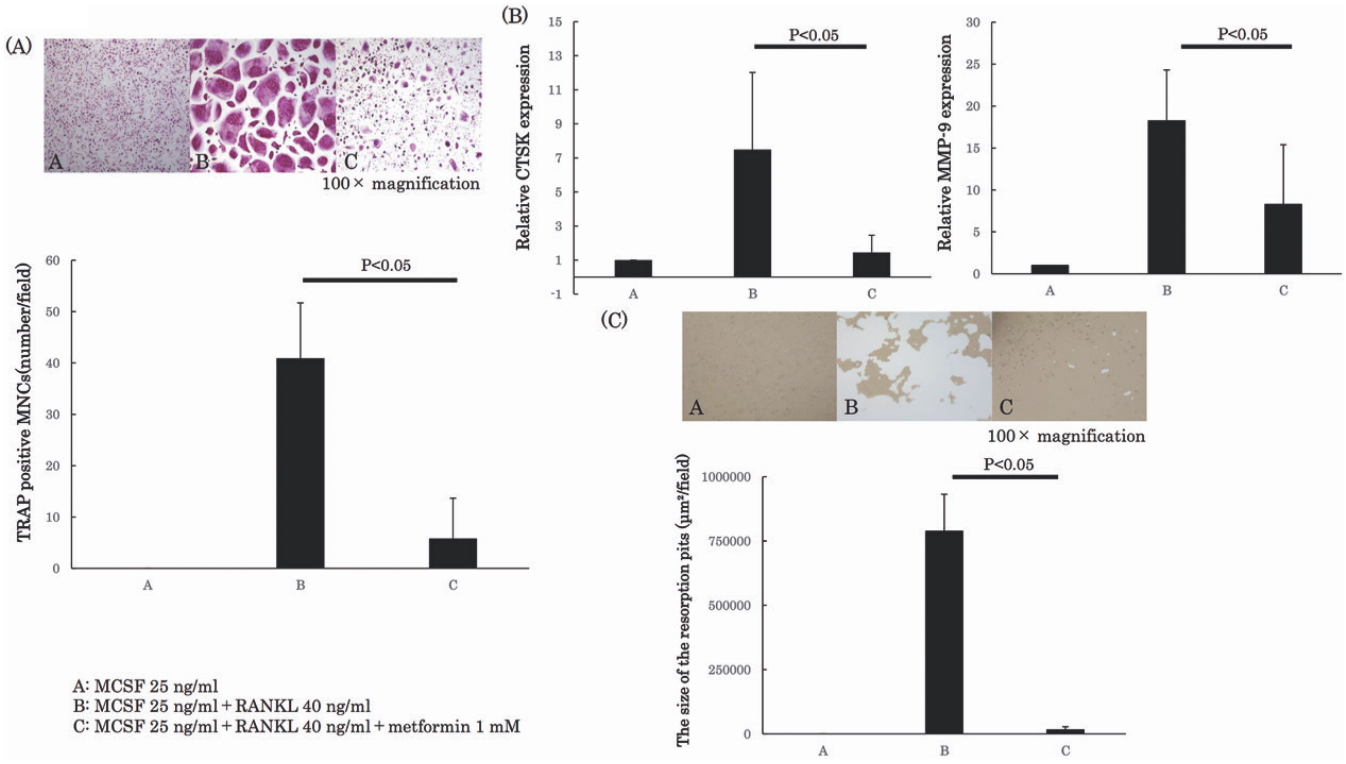


Fig. 1. Metformin inhibits M-CSF/RANKL-induced osteoclastogenesis.
$CD14^+$ cells from healthy donors were treated with indicated reagents for 7 days.
A: Osteoclasts observed in TRAP-positive (red) and multinucleated (> 5 nuclei) cells. Representative photomicrographs for TRAP staining. Scale bars, 200 μm. Bar graph showing numbers of osteoclasts.
B: Comparison of the mRNA expression of enzymes involved in joint destruction (cathepsin K (CTSK; left panel) and MMP-9 (right panel)) during osteoclastogenesis evaluated by quantitative real-time PCR. All mRNA expression levels were normalised to that of β-actin.
C: $CD14^+$ cells cultured in a Corning Osteo Assay Surface microplate with indicated regents for 7 days. Representative photomicrographs for bone resorption pits. Scale bars, 200 μm. Bar graph showing the quantification of the resorption area. The data shown are representative of three different experiments. All bar graphs are expressed as mean ± SD.

destruction, were reduced in TNF-α-stimulated MH7A following treatment with metformin (Fig. 2A).

Thus, we examined whether metformin suppressed the TNF-α-induced expression of insulin -like growth factor (IGF)-1 and IGF-1 receptor (R) in MH7A cells. As shown in Figure 2B, treatment with TNF-α led to the markedly induced expression of IGF-1 and IGF-1R, whereas the expression of these genes was shown to be reduced in metformin-treated MH7A cells.

Metformin tended to disrupt endothelial tube formation

To investigate whether addition of metformin resulted in the suppression of angiogenesis, we performed a tube-forming assay using HUVEC cells with or without treatment with metformin. Our results revealed that treatment of HUVEC cells with metformin tended to reduce the formation of tubular net-

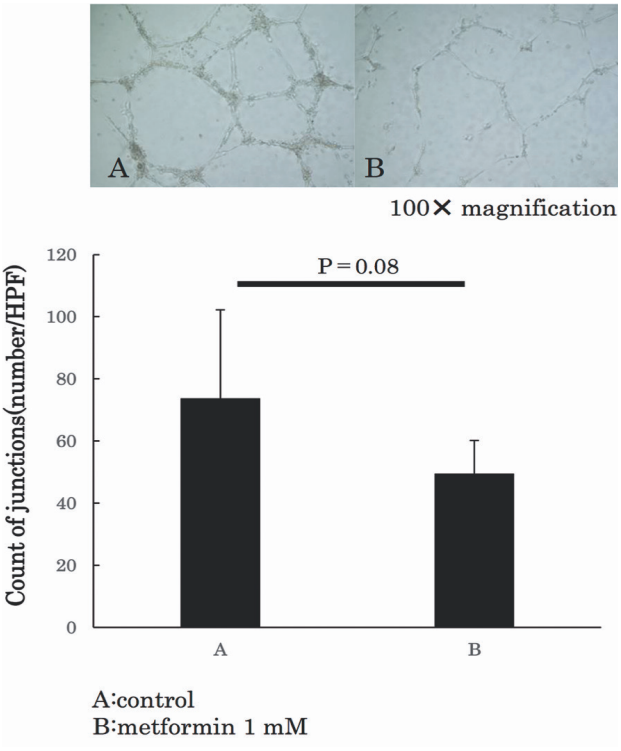
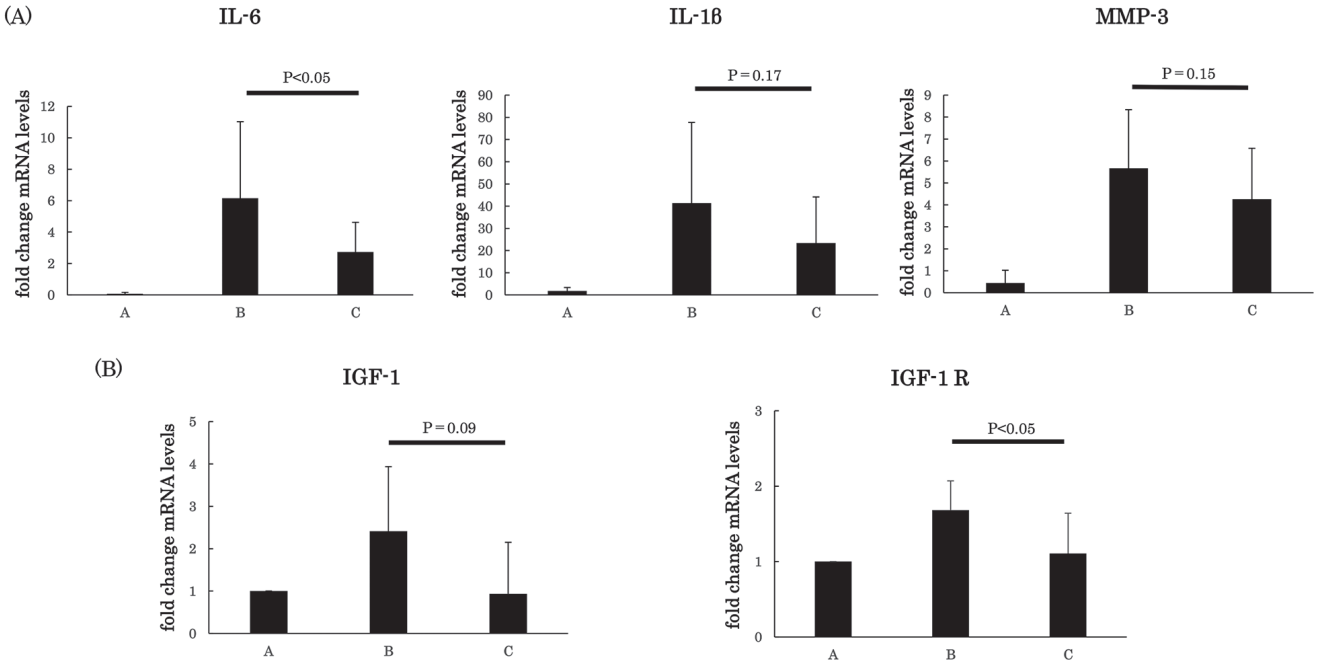


Fig. 3. Metformin tends to reduce angiogenesis.
HUVEC cells seeded with or without metformin on Matrigel precoated wells for 14 h. Representative images of HUVEC tube formation are shown.
Scale bars, 100 μm.
Bar graph showing the number of quantified junctions.
The data shown are representative of three different experiments.
Data are expressed as the mean ± SD.



A: control
B: TNF-α 10 ng/ml
C: TNF-α 10 ng/ml + metoformin 1 mM

Fig. 2. Effect of metformin on the TNF-α-induced inflammatory response in a human synovial fibroblast cell line.
The MH7A human synovial cell line was pretreated with or without metformin for 1 h, and exposed to TNF-α for 24 h. Expression of (**A**) IL-6, IL-1β, and MMP-3 and (**B**) IGF-1 and IGF-1R determined by quantitative real-time PCR. The data shown are representative of three different experiments. Data are expressed as the mean ± SD.

works (Fig. 3), indicating that metformin could suppress angiogenesis.

Discussion

Metformin is known as a drug used in the treatment of type 2 diabetes. Recently, there have been reports suggesting the efficacy of metformin in the treatment of various types of cancer, cardiovascular disease, aging, and neuropathy (10). More recently, several studies in autoimmune diseases have shown that metformin was able to regulate various cells involved in autoimmune disorders and restore immune homeostasis. As a result, disease activity was shown to be improved in different murine models of autoimmunity (11). Recent research showed that metformin was able to improve the pathology of mice with CIA by suppressing osteoclastogenesis through the AMPK signalling (12). Noted, AMPK has been reported to sense the decrease in adenosine triphosphate (ATP) induced by energy stress resulting in its phosphorylation. Then, the phosphorylated AMPK has been shown to promote catabolism and suppress anabolic metabolism to increase ATP and restore energy (12). Activated AMPK has also been reported to have another role, which is to suppress the mammalian target of rapamycin (mTOR). By activation of translation in response to amino acids, growth factors, mitogens and insulin, mTOR has been demonstrated to promote anabolic metabolism and suppress catabolic metabolism, resulting in cell growth and proliferation (13).

Our data showed that metformin inhibited osteoclastogenesis and the function of osteoclasts, including bone resorption (Fig. 1). These results were consistent with a previous study using mice with CIA (5). Therefore, it was suggested that metformin could inhibit bone destruction in human RA, and similar to mice with CIA, metformin-induced activation of AMPK might have contributed to this effect. Our findings indicated that metformin could suppress bone destruction in RA.

Metformin has been reported to suppress synovial cell proliferation (14). Furthermore, our previous reports showed that IGF-1 signalling contributed to RA aggression (7, 8). In this study, we reported the anti-inflammatory effect of metformin in a synovial cell line. In particular, metformin was shown to reduce the TNF-α-stimulated induction of IL-6 in MH7A cells (Fig. 2A). The activation and proliferation of synovial cells are known to be autocrinally stimulated by inflammatory cytokines, including IL-6. Moreover, activated synovial cells have also been shown to produce many bioactive substances, including MMPs. These molecules are known to generate synovial thickening and joint destruction (15). The MH7A human RA synovial fibroblast cell line has been reported to express various inflammatory genes upon stimulation by TNF-α (6, 9). More specifically, TNF-α-stimulated synovial cells have been shown to secrete IL-6, stimulating the autocrine function and inducing tumour-like proliferation of synovial cells (15). Furthermore, we observed that the TNF-α-induced expression of IGF-1 and IGF-1R was reduced by treatment with metformin (Fig. 2B). Our previous findings showed that blocking of the IGF-1 Ab signalling by anti-IGF-1R and IGF 1R inhibitor reduced the

de novo expression of IGF-1 (7) and synoviocyte proliferation (8), respectively. Taken together, suppression of the expression of IGF-1 and IGF-1R by metformin might contribute to the inactivation of synoviocytes. Therefore, metformin-induced repression of the autocrine expression of these growth factors in inflammatory synoviocytes could suppress RA pathology. Human RA pannus is known to be formed by inflammatory cell infiltration, synovial proliferation and angiogenesis (16-18). Excessive proangiogenic factors have been reported to lead to elevated synovitis and pannus formation. Conversely, inhibition of joint neovascularisation has been shown to be able to alleviate them (19). In this study, our data showed that metformin tended to disrupt HUVEC tube formation (Fig. 3). The formation of tubes produced by endothelial cells is known to be an important step during neoangiogenesis (20). Angiogenesis has been observed in the synovial tissue pathology of patients with active RA and has been reported to play a key role in maintaining synovitis (18). Previous report demonstrated that metformin had antiangiogenic effects on another type of endothelial cells, human retinal vascular endothelial cells, although the precise mechanism remains unknown (21). Thus, our *in vitro* study using synovial and endothelial cells suggested that metformin might suppress pannus formation in human RA.

In conclusion, we investigated the possibility that metformin might attenuate human RA. Our results showed that metformin suppressed RANKL/M-CSF-induced osteoclastogenesis in MH7A under conditions of an inflammatory response and HUVEC tube formation *in vitro* using human cells. According to these results and the previous report on a mouse model of CIA (5), metformin might be a promising drug in improving human RA *in vivo*. The precise mechanism of the function of metformin on RA requires further investigation, and if achieved, could lead to drug repositioning of metformin.

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