Inhibitory effects of anti-rheumatic drugs on vascular endothelial growth factor in cultured rheumatoid synovial cells

M. NAGASHIMA, S. YOSHINO, H. AONO*, M. TAKAI* & M. SASANO* *Department of Joint Disease and Rheumatism, Nippon Medical School, Tokyo, and *Developmental Research Division, Santen Pharmaceutical Co. Ltd, Osaka, Japan*

SUMMARY

Vascular endothelial growth factor (VEGF) is a potent inducer of angiogenesis and is constitutively expressed in the synovium of rheumatoid arthritis (RA). Over-expression of VEGF may play an important role in pathogenic vascularization and synovial hyperplasia of RA. In the present study, we examined whether disease-modifying anti-rheumatic drugs (DMARDs), including bucillamine (BUC), gold sodium thiomalate (GST), methotrexate (MTX) and salazosulfapiridine (SASP), act by inhibiting the production of VEGF by cultured synovial cells of patients with RA. Treatment of cultured synoviocytes with lipopolysaccharide (LPS) significantly increased VEGF production by cultured synovial cells. BUC significantly inhibited LPS-induced VEGF production, while GST tended to inhibit the production of VEGF. The inhibitory effects on VEGF production were dose-dependent. In contrast, MTX and SASP did not affect VEGF production. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed that BUC also inhibited LPS-induced VEGF mRNA expression in RA synovial cells. The present study provides the first evidence that BUC inhibits VEGF production and the expression of its mRNA in synovial cells of RA patients. Our results indicate that the anti-rheumatic effects of BUC are mediated by suppression of angiogenesis and synovial proliferation in the RA synovium through the inhibition of VEGF production by synovial cells.

Keywords rheumatoid arthritis   synoviocytes   vascular endothelial growth factor   disease-modifying anti-rheumatic drugs   angiogenesis

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory and systemic autoimmune disease characterized by hyperplasia of synovial cells and angiogenesis, which ultimately lead to the destruction of cartilage and bone. Angiogenesis, the growth and proliferation of new blood vessels, is important in a variety of pathophysiologic processes in the RA synovium. A number of angiogenic factors are involved in the neovascularization process in the RA joint. These include acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), platelet-derived endothelial cell growth factor and vascular endothelial growth factor (VEGF). These growth factors stimulate vascular endothelial cells in autocrine and paracrine manners [1,2].

VEGF is present in normal tissues [3–6] and certain neoplastic tissues [7–9]. It is a unique peptide growth factor that specifically stimulates the proliferation of endothelial cells [10] and is thought to play an essential role in angiogenesis during a variety of

biological processes, including tissue repair and regeneration [11], and tumour growth [8,12,13]. VEGF is also a potent inducer of microvascular extravasation [14,15]. We have previously reported that VEGF polypeptide and mRNA are distributed in the perivascular space and that both are expressed by subsynovial macrophages and synovial lining cells in the synovial tissues of RA patients using immunohistochemical staining, *in situ* hybridization and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis [16]. In addition, cultured synovial cells are also known to express VEGF under hypoxic conditions or stimulation by IL-1 [17]. Thus, these observations suggest that the constitutive expression of VEGF in rheumatoid synovial cells may play an important role in the pathophysiology of RA synovium.

Several disease-modifying anti-rheumatic drugs (DMARDs) have been used to control RA. While the majority of these DMARDs act as immunomodulatory drugs in RA [18–25], some also act by inhibiting the angiogenic process [26–31]. However, the mechanism of the inhibitory effects of DMARDs on angiogenesis remains obscure.

We speculated that DMARDs inhibit angiogenesis in the synovium of RA by suppressing VEGF production and VEGF

Correspondence to: Masakazu Nagashima MD, PhD, Department of Joint Disease and Rheumatism, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan.

mRNA expression in synovial cells. In the present study, we examined the effect of bucillamine (BUC), gold sodium thiomalate (GST), methotrexate (MTX) and salazosulfapiridine (SASP) on the production of VEGF by cultured synovial cells of RA patients.

PATIENTS AND METHODS

Patients and cell preparation

Tissue specimens were obtained from eight patients with RA (stage III or IV) who fulfilled the diagnostic criteria of the American College of Rheumatology with a disease duration of 10–15 years. For comparative analysis, we also obtained tissues from four patients with osteoarthritis (OA). After informed consent, synovial tissue samples were obtained from patients with RA and OA during synovectomy of the knee or total knee joint arthroplasty. The synovial samples were immediately prepared as described previously [32,33]. Briefly, the synovial tissue was cut into small pieces, washed three times in PBS, and treated with 1 mg/ml collagenase (Sigma Chemical Co., St Louis, MO) for 30–60 min at 37°C. The cells were suspended in Ham F-12 medium (Nikken Bio Medical Lab., Kyoto, Japan) containing 10% fetal calf serum (FCS; Flow Labs, McLean, VA), 100 U/ml penicillin and 100 μg/ml streptomycin. The cell suspension was plated onto 90-mm culture dishes and cultured in a humidified 5% CO_2 incubator. When cell cultures reached confluence, synovial cells were treated with trypsin and further passaged to other dishes. The cells used in the present experiments were from passages two to five.

DMARDs

BUC and SA981 (a metabolite of BUC) were obtained from Santen Pharmaceutical Co. (Osaka, Japan). SASP, GST, MTX and dexamethasone (DEX) were obtained from Sigma, Shionogi Co. (Osaka, Japan), Nacalai Tesque (Kyoto, Japan) and Biomal Res. Lab. (Plymouth Meeting, PA), respectively. BUC, GST and SASP were used at concentrations ranging from 1 to 100 μg/ml, while those of MTX and DEX ranged from 0·1 to 10 μg/ml and 1 ng/ml to 1 μg/ml, respectively.

These concentrations of DMARDs *in vitro* were decided according to those *in vivo* [34–37] and the concentrations *in vitro* were about from 10–30-fold those *in vivo*.

Analysis of VEGF concentration in culture supernatants of synovial cells

For the assay of VEGF production, 24-well flat-bottomed microtitre plates were seeded with 5×10^4 cells in the culture medium. After 24 h, cell growth was arrested by replacing the culture medium with 1% FCS in Ham F-12. In the next step, lipopolysaccharide (LPS; Difco Labs, Detroit, MI) with or without DMARDs was added, followed by incubation for another 72 h, and collection of the supernatants at the end of the culture period. The concentration of VEGF in the supernatant was measured by using VEGF ELISA kits (Immuno-Biological Lab Co., Gunma, Japan) and absorbance at 450 nm was recorded using an ELISA plate reader (BioRad, Richmond, CA).

Detection of VEGF mRNA expression by RT-PCR

After washing cultured synovial cells with PBS (−), they were stimulated with 10 μg/ml LPS for 24 h, then harvested and used for the extraction of total RNA using Isogen (Nippon Gene, Tokyo, Japan). Poly A mRNA was isolated using a poly AT tract mRNA isolating kit (Promega, Madison, WI). The first strands of cDNA were synthesized from 2 μg of polyadenylated RNA using the First Strand cDNA synthesis kit (Boehringer, Mannheim, Germany). Oligonucleotide primers were produced by a DNA synthesiser according to the human VEGF cDNA sequence (GeneBank no. M32977]: primer 1, 72–92 (5′-TCTTGGGTGCATTG-GAGCCTC-3′); and primer 2, 421–401 (5′-AAGCT-CATCTCTCCTATGTGC-3′). RT-PCR was performed using a previously described technique [33] with some modifications. For this purpose, 50-μl aliquots of the reaction mixture were prepared, containing 5 μl of 10× PCR buffer (Takara, Shiga, Japan), 300 ng of template cDNA, and 100 pmol of each of 5′ and 3′ primers. PCR assays for VEGF and β-actin were performed in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 30 cycles, each consisting of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. The PCR products were analysed on a 1·5% agarose gel and the density of each band was analysed semiquantitatively using a densitometer.

Proliferation assay by MTS

MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay kits (Promega) was used as non-radioactive cell proliferation assays. For this purpose, synovial cells were plated at 1×10^4/well onto 96-well plates (third passage) and incubated for 24 h in Ham F-12 medium containing 10% FCS (Flow Labs) at 37°C in a humidified 5% CO_2 atmosphere. The culture media were replaced with F-12 medium containing 1% FCS and incubated for 72 h at 37°C. Following the addition of 20 μl/well of a freshly prepared MTS/PMS solution (100 μl of PMS solution was combined with 2·0 ml of MTS solution immediately before addition to the culture plate containing the cells), the plate was incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere. This was followed by measurement of absorbance at 490 nm using an ELISA plate reader (BioRad).

Statistical analysis

All values were expressed as mean ± s.e.m. Differences between groups were tested for statistical significance using Student's *t*-test and Sheffe's test. $P < 0.05$ denoted the presence of a significant difference.

RESULTS

Inhibition of VEGF production in the culture supernatant

LPS, as well as a variety of other agents, are potent stimuli for IL-1β, IL-6 and tumour necrosis factor-alpha (TNF-α) release by synovial cells [38,39]. We first examined whether LPS activates the production of VEGF on synovial cells of patients with RA and OA. After culture of synovial cells in 1% FCS in Ham F-12, we added 10–100 μg/ml LPS and incubated the cells for 72 h. The concentration of VEGF in the supernatants of synovial cell cultures was measured using VEGF ELISA kits. LPS induced the production of VEGF in synovial cells in a dose-dependent manner (data not shown). As shown in Fig. 1, 10 μg/ml LPS caused a two-fold increase in the production of VEGF by synovial cells (Fig. 1). LPS-enhanced production of VEGF was time-dependent, extending between 4 h and 72 h.

Next, we examined the effects of DMARDs on LPS-induced production of VEGF by synovial cells. After culture of synovial cell in 1% FCS in Ham F-12 and 10 μg/ml LPS, variable concentrations of DMARDs were added, followed by incubation of the
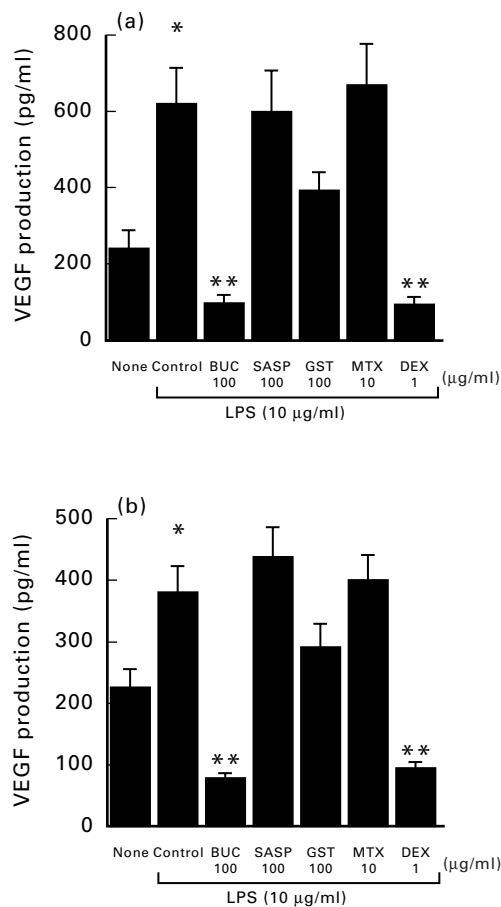
Fig. 1. Effect of disease-modifying anti-rheumatic drugs (DMARDs) on vascular endothelial growth factor (VEGF) production by lipopolysaccharide (LPS)-stimulated synovial cells from four patients with osteoarthritis (OA) (a) and eight patients with rheumatoid arthritis (RA) (b). Results represent the mean ± s.e.m. *$P < 0.05$ compared with unstimulated untreated synoviocytes (none); **$P < 0.01$ compared with control synoviocytes. BUC, Bucillamine; SASP, salazosulfapyridine; GST, gold sodium thiomalate; MTX, methotrexate; DEX, dexamethasone.

cells for another 72 h. LPS-stimulated high VEGF production by cultured synovial cells of patients with RA was inhibited by BUC and DEX, to 78.0 ± 8.2 pg/ml and 93.0 ± 11.2 pg/ml, respectively (Fig. 1). Furthermore, BUC and DEX also inhibited LPS-stimulated VEGF production by OA synoviocytes to 98.8 ± 20.5 pg/ml and 97.0 ± 18.2 pg/ml, respectively (Fig. 1). These values were significantly different from those of DMARD-untreated stimulated synovial cells (380.7 ± 43.2 pg/ml). GST tended to inhibit the production of VEGF by cultured synovial cells, albeit insignificantly. In contrast, MTX and SASP did not inhibit VEGF production by cultured synovial cells of RA patients.

In the next step, we investigated the dose response to BUC, MTX and DEX. As shown in Fig. 2, BUC and DEX inhibited VEGF production in a concentration-dependent manner. BUC significantly inhibited VEGF production from 419.6 ± 17.2 pg/ml (stimulated by LPS 10 μg/ml) to 203.2 ± 14.2 pg/ml when used at a concentration of 30 μg/ml. Similarly, DEX significantly inhibited VEGF production to 156.58 ± 2.47 pg/ml when used at a dose of 0.01 μg/ml.

BUC is a thiol compound that contains two free sulfhydryl groups and differs from D-penicillamine (D-Pen). It has been
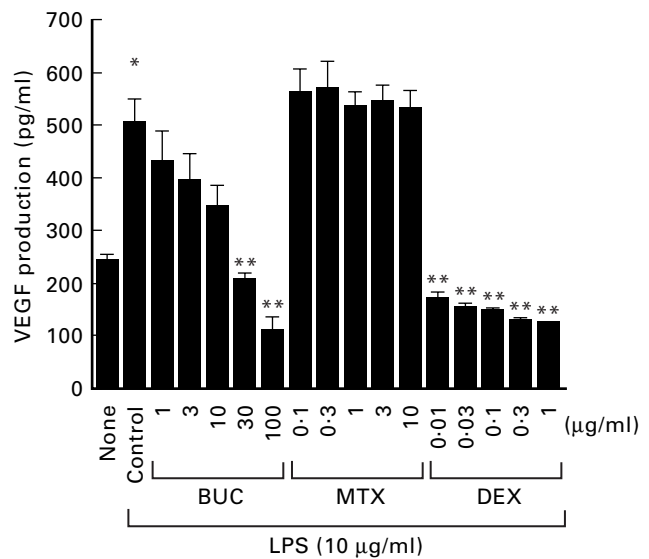

Fig. 2. Inhibitory effect of bucillamine (BUC), methotrexate (MTX) and dexamethasone (DEX) on vascular endothelial growth factor (VEGF) production by lipopolysaccharide (LPS)-stimulated synovial cells. Results represent the mean ± s.e.m. of eight patients with rheumatoid arthritis (RA). *$P < 0.05$ compared with unstimulated untreated synoviocytes (none); **$P < 0.01$ compared with control synoviocytes.

reported that SA981, a metabolite of BUC with an intramolecular disulphide, has greater effects on B and T cell function than BUC itself [18,41]. In the next step, we investigated whether other thiol compounds such as SA981 and D-Pen exert an inhibitory effect on VEGF production by LPS-stimulated cultured synovial cells. Both BUC (93.3 ± 12.3 pg/ml) and SA981 (169.8 ± 19.4 pg/ml) significantly inhibited VEGF production compared with control cultured synovial cells (466.0 ± 73.3 pg/ml). Furthermore, these effects were concentration-dependent. In contrast, D-Pen did not influence VEGF production (data not shown).

RT-PCR assay of cultured synovial cells
We also examined the effect of DMARDs on VEGF mRNA expression on LPS-stimulated cultured synovial cells using RT-PCR. After treatment for 24 h, total RNA was extracted from
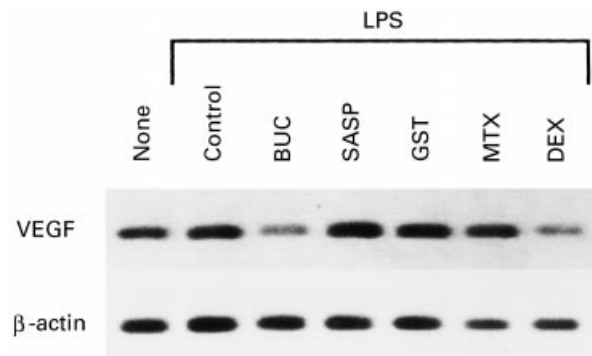

Fig. 3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of vascular endothelial growth factor (VEGF) and β-actin. Note that bucillamine (BUC) as well as dexamethasone (DEX) showed an inhibitory effect on VEGF mRNA.
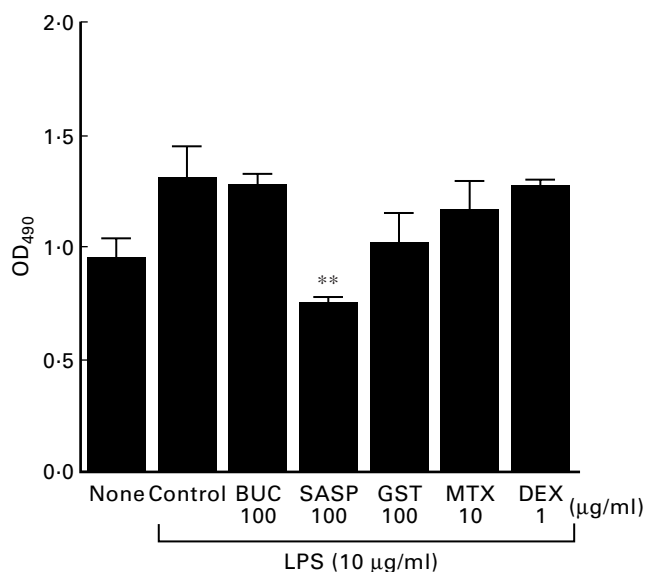
Fig. 4. Effect of disease-modifying anti-rheumatic drugs (DMARDs) on the proliferation of lipopolysaccharide (LPS)-stimulated synovial cells. Results represent the mean ± s.e.m. of eight patients with rheumatoid arthritis. **$P < 0.01$ compared with control synoviocytes.

the synovial cells and converted to cDNA by RT. A semiquantitative RT-PCR assay was used for the detection of VEGF mRNA, relative to β-actin mRNA expression, as described previously [33]. As shown in Fig. 3, over-expression of VEGF mRNA was detected in LPS-stimulated synovial cells. BUC and DEX, but not MTX and SASP, inhibited VEGF mRNA expression.

MTS assay of cultured synovial cells
To investigate whether DMARDs suppressed VEGF production by inhibition of proliferation of cultured synovial cells, we used the MTS assay to examine the effect of DMARDs on the proliferation of these cells. As shown in Fig. 4, all DMARDs tested in the present study, with the exception of SASP, had no effects on synovial cell proliferation. Thus, the inhibitory effects of DMARDs on VEGF production noted above were not due to inhibition of the proliferation of synovial cells, but rather due to inhibition of VEGF polypeptide or VEGF mRNA.

DISCUSSION

The major findings of the present study were the inhibitory effects of DMARDs on VEGF production in both RA and OA. Furthermore, BUC and DEX exerted significant inhibitory effects on VEGF production in a dose-dependent manner. These inhibitory effects were not due to cytotoxic effects or inhibition of proliferation of synovial cells, but rather involved the production or mRNA expression of VEGF. Our results also showed that MTX and SASP had no inhibitory effects on VEGF production even when used at 10-fold concentrations. On the other hand, only a marginal inhibitory effect was noted for GST with regard to the production of VEGF by cultured synovial cells. Our results showed a superior effect for BUC than other DMARDs, and thus it may be the most useful DMARD for inhibition of VEGF production *in vivo*. It has been reported that the maximal plasma concentration of BUC is 10^{-5}–10^{-6} M after oral administration of 300 mg BUC,

and the concentration in tissues, such as synovium, joint cartilage and other collagen-rich tissues, is higher than that in plasma [40]. It is suggested that the inhibition of VEGF production by BUC in this study possibly occurs in the synovium of treated patients.

BUC is a thiol compound that contains two free sulfhydryl groups, thus making it different from D-Pen. It has been reported that SA981, a metabolite of BUC with an intramolecular disulphide, has greater effects on B cell and T cell function than BUC itself [18,41]. Therefore, we examined the effects of thiol compounds such as SA981 and D-Pen on VEGF production by cultured synovial cells. Our results showed that both BUC and SA981, but not D-Pen, significantly inhibited VEGF production by cultured synoviocytes in a concentration-dependent manner (data not shown). Matsubara *et al.* [28,31] reported that D-Pen inhibited IL-1-induced synovial and endothelial cell proliferation in the presence of copper sulphate. This observation indicates that hydrogen peroxide produced by D-Pen together with copper inhibits angiogenesis *in vivo*, and IL-1-induced synovial and endothelial cell proliferation. Therefore, we investigated whether hydrogen peroxide by BUC and SA981 may play a central role in inhibition of VEGF production by thiol compounds. However, the inhibitory effect of BUC did not affect the production of VEGF with or without catalase (data not shown). Thus, these findings suggest the inhibitory effects of BUC and SA981 in synovial cells are not due to the production of hydrogen peroxide.

VEGF is composed of four polypeptides, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$, produced by alternative splicing of VEGF mRNA. These polypeptides possess a signal peptide composed of 26 amino acids. In particular, $VEGF_{165}$ and $VEGF_{121}$ exert dose-dependent mitogenic effects on vascular endothelial cells [6]. On the other hand, despite the presence of a signal peptide, the 24 amino acid insertion common to $VEGF_{189}$ and $VEGF_{206}$ apparently prevents effective secretion of these polypeptides. Therefore, $VEGF_{165}$ and $VEGF_{121}$ are considered to be present mainly in the culture supernatant, while $VEGF_{189}$ and $VEGF_{206}$ are present mainly in cell lysates. Our results show that BUC and DEX inhibited the production of VEGF polypeptide by cultured synovial cells and synovial cell lysates. On the other hand, DEX inhibited VEGF production in rat glioma [42] and similar results were also demonstrated in cultured synovial cells. DEX also showed a significant inhibitory effect at a concentration exceeding that *in vivo* [43,44]. In this regard, it has also been reported that DEX down-regulates the induced expression of VEGF gene, suggesting a transcriptional repression mechanism within the promotor of the VEGF gene [45]. These findings suggest that VEGF antagonists, that prevent the interaction between VEGF and its receptor, may be beneficial in RA. Further studies are required to understand the mechanisms of the inhibitory effects of BUC on VEGF mRNA expression and production.

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