member of this group of SPB- and horsetail-movement-associated proteins. In support of this, *mcp6Δ* cells show similar phenotypes to the *kms1-1*, *kms1Δ*, *dhc1* and *dlc1Δ* mutant cells as follows. First, like *kms1*, *dhc1* and *dlc1Δ* mutants, the rates of intergenic or intragenic recombination of the *mcp6Δ* mutant are markedly reduced (Fig. 5A,B). Second, the ectopic recombination rate of *mcp6Δ* cells is increased about twofold (Fig. 5C), as has also been observed for the ectopic recombination of *kms1Δ* cells between *ade6-M26* and *ade6-469* (*z15*) loci, although the rate of ectopic recombination between the *ade6-M26* and *ade6-469* (*z7*) loci in *kms1Δ* cells was almost the same as that in wild-type cells (Niwa et al., 2000).

However, Mcp6 does have some phenotypes that differ from those of *kms1*, *dhc1* and *dlc1Δ* mutants, and thus it seems to play a distinct role in maintaining the horsetail movement. One such phenotype is the almost normal spore formation and spore viability in *mcp6Δ* cells (Fig. 5D,E). By contrast, spore formation and spore viability are abnormal in *kms1Δ*, *dhc1Δ* and *dlc1Δ* cells. Second, unlike *kms1Δ* cells (Shimanuki et al., 1997), telomere clustering, as determined by examining the subcellular localization of Taz1-GFP and Swi6-GFP, appears almost normal in *mcp6Δ* cells (Fig. 8). Third, although *kms1Δ* and *dhc1-d3* cells showed reduced SPB integrity and abnormal chromosome segregation, respectively, the *mcp6Δ* mutant does not show such abnormalities (Figs 6, 7). Fourth, Mcp6 is the only meiosis-specific protein of this group, because Kms1, Dhc1 and Dlc1 are also detected in both the mitotic and meiotic phases (Goto et al., 2001; Miki et al., 2002).

Mcp6 plays a role in homologous pairing by regulating horsetail movement

We report here that recombination rates are greatly reduced in *mcp6Δ* cells compared with wild-type cells (Fig. 5), which is due primarily to the inefficient homologous pairing of chromosomes in *mcp6Δ* cells (Fig. 4). This inefficient homologous pairing is mainly derived from the impaired horsetail movement of nucleus (Fig. 3). We previously showed that *meu13Δ* and *mcp7Δ* cells show a delay in entering meiosis I, and that this is due to the meiotic recombination checkpoint that provides cells with enough time to repair double-strand breaks (DSBs) in a manner dependent on checkpoint *rad*[+] genes (Shimada et al., 2002; Saito et al., 2004). In the case of *mcp6Δ* cells, however, even though recombination rates were reduced, a delay in entering meiosis I was not observed (Fig. 3A). Thus, we surmise that Mcp6 is not involved in DSB repair like other SPB components, probably because Mcp6 is located at the SPB, a location that is too remote directly to regulate DSBs on chromatin. Similarly, Mcp6 might not directly regulate the recombination machinery. Thus, it is most probable that Mcp6 is involved in regulating the pairing of homologous chromosomes by inducing proper horsetail movement.

Mcp6 is a novel type of regulator of horsetail movement

Some mutants (*lot2-s17*, *lot3-uv3/tazl*, *dot1* and *dot2*) show defective horsetail movement and reduced meiotic recombinations, but their phenotypes are distinct from *mcp6Δ*. For example, *lot2-s17* and *lot3-uv3/tazl* mutants display a

dramatic lengthening of telomeric repeats, low spore viability and chromosome mis-segregation through meiosis (Nimmo et al., 1998; Cooper et al., 1998; Hiraoka et al., 2000). Two mutants (*dot1* and *dot2*) do not sporulate and show defective SPB integrity and impaired telomere clustering (Jin et al., 2002). By contrast, we show here that *mcp6Δ* differs from these mutants because sporulation (Fig. 5D,E), SPB integrity (Fig. 7) and telomere clustering (Fig. 6) are almost normal.

Some mutant strains of *taz1* that show abnormal horsetail movement (Hiraoka et al., 2000) are also distinct from *mcp6Δ* cells. First, telomere clustering is abnormal in *taz1Δ* cells. Second, *taz1Δ* cells display abnormal spore formation and reduced spore viability. Third, although the subcellular localization of Taz1-GFP is normal in *mcp6Δ* cells (Fig. 8A), Mcp6-GFP localization became slightly abnormal in *taz1Δ* cells (Fig. 6A,C). Thus, Mcp6 might interact with Taz1 but its role in chromosome maintenance during meiosis is quite distinct.

Finally, microtubules are nucleated exclusively from SPBs immediately after karyogamy and form typical X-shaped configurations during nuclear fusion of meiotic cells (Svoboda et al., 1995; Yamamoto et al., 1999). However, we report here that the astral microtubule organization was largely abnormal in *mcp6Δ* cells (Fig. 8). Thus, in the absence of Mcp6, many cells fail to organize a long, curved microtubule array that extends from the cell ends to provide the tracks for nuclear horsetail movement, resulting in the abolished nuclear oscillation and reduced chromosome pairing. Taken together, we conclude that Mcp6 controls horsetail movement by regulating the astral microtubule organization during meiosis.

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Inhibitory effects of bucillamine on the expression of vascular cell adhesion molecule-1 in human umbilical vein endothelial cells

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Abstract

Bucillamine (BUC) has been found to have beneficial effects in the treatment of rheumatoid arthritis (RA), in which the activation of endothelial cells plays an important role in the pathogenesis. The current studies examined the effect of BUC and its intramolecular disulfide form (BUC-ID) on the expression of adhesion molecules in human umbilical vein endothelial cells (HUVEC) stimulated with tumor necrosis factor-α (TNF-α). HUVEC (4×10^4/well) were incubated with medium M199 containing heparin and 20% FCS with endothelial cell growth supplement (ECGS) for 24 h in the presence or absence of BUC or BUC-ID, after which the culture medium was replaced with ECGS free medium. Then the cultures were further carried out for additional 24 h with TNF-α (10 ng/ml) in the presence or absence of BUC or BUC-ID. BUC-ID, but not BUC, appeared to suppress the expression of VCAM-1 on HUVEC stimulated with TNF-α in a dose-response manner at its pharmacologically relevant concentrations (0.3–3.0 μg/ml), whereas only the 3 μg/ml concentration level of BUC-ID had a statistically significant effect, although the effect was relatively small. By contrast, lower concentrations of BUC-ID (1–3 μg/ml) suppressed the secretion of soluble VCAM-1 by HUVEC much more effectively. Of note, at the concentration of 3 μg/ml neither BUC nor BUC-ID significantly influenced the expression of ICAM-1 and E-selectin on TNF-α stimulated HUVEC. These results indicate that BUC-ID, but not BUC, specifically downregulates the surface expression of VCAM-1 as well as the release of soluble VCAM-1 by HUVEC stimulated with TNF-α. BUC-ID suppressed the production of solubleVCAM-1 by RA bone marrow CD34+ cells stimulated with SCF, GM-CSF and TNF-α more effectively than BUC. The data thus suggest that one of the mechanisms of action of BUC involves the inhibition of the activation of endothelial cells.
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Keywords: Rheumatoid arthritis; Bucillamine; Adhesion molecules; Endothelial cells; TNF-α; VCAM-1; ICAM-1; E-selectin

1. Introduction

Bucillamine [*N*-(2-mercapto-2-methylpropionyl)-L-cysteine] (BUC) is a disease-modifying antirheu-

matic drug [1]. The beneficial effects of BUC in the treatment of rheumatoid arthritis (RA) have been well appreciated in several clinical trials [1–3]. BUC is a thiol compound that differs from D-penicillamine (DP) by the presence of two free sulfhydryl groups. As a result, a considerable fraction of BUC can form an intramolecular disulfide (BUC-ID) that appears to have unique immunosuppressive activities [4]. Thus, BUC exerts immunosuppressive effects

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that are similar to those of DP, as well as unique inhibitory effects that depend upon the capacity of BUC to form an intramolecular disulfide, BUC-ID [4]. Moreover, we have also delineated that at pharmacologically attainable concentrations, BUC-ID directly suppresses B cell IgM production, but not T cell interferon-γ (IFN-γ) production, whereas DP inhibits the latter, but not the former, suggesting that as a result of the formation of the internal disulfide, BUC and DP have different targets of immunosuppressive action in vivo [5].

The hallmarks of the pathological changes in the RA synovium include hyperplasia of synovial lining cells and follicle-like aggregation of lymphocytes and plasma cells [6]. A number of studies have indicated that angiogenesis as well as activation of endothelial cells to express adhesion molecules plays a crucial role in recruiting inflammatory cells and immunocompetent cells into the synovium [7–9]. Of note, BUC has been shown to inhibit the production of vascular endothelial growth factor (VEGF), a potent inducer of angiogenesis, by cultured rheumatoid synovial cells [10] and bovine retinal microcapillary endothelial cells [11]. However, the effects of BUC and its metabolites on the expression of adhesion molecules in vascular endothelial cells have not yet been delineated. The current studies therefore examined the effects of BUC and BUC-ID on the expression of adhesion molecules in human umbilical vein endothelial cells (HUVEC) stimulated with TNF-α.

2. Materials and methods

2.1. Monoclonal antibodies (mAb) and reagents

A variety of mAb were used, including a murine IgG1 mAb directed at VCAM-1 (CD106) (clone 1G11; Immunotech, Marseille, France), a murine IgG1 mAb directed at ICAM-1 (CD54) (clone 84H10, Immunotech), a murine IgG1 mAb directed at E-selectin (CD62E) (clone 1.2B6, Southern Biotechnology Associates, Birmingham, AL) and a murine IgG1 control mAb (MOPC21) (Cappel Laboratories, West Chester, PA). TNF-α was purchased from PeproTech, London, UK. Bucillamine (BUC) and its intramolecular disulfide (BUC-ID)

were synthesized and provided by Santen Pharmaceutical, Osaka, Japan.

2.2. Preparation of HUVEC cultures

HUVEC were isolated from single harvests in our laboratory as described [12]. HUVEC were maintained in M199 medium (Life Technologies, Grand Island, NY) supplemented with 20% FCS (Life Technologies), 100 μg/ml heparin, 50 μg/ml endothelial cell growth supplement (ECGS) (Becton Dickinson Labware, Bedford, MA), 2 mM L-glutamine, penicillin G (100 U/ml), streptomycin (100 μg/ml), and 25 mM HEPES (Life Technologies) in culture flasks coated with 20 μg/ml fibronectin (Ito ham, Tokyo, Japan). HUVEC were used at passages 3–5 for all the experiments.

2.3. Cell enzyme-linked immunosorbent assay (cell ELISA)

HUVEC (4×10^4/well) were incubated with medium M199 containing heparin and 20% FCS with ECGS in wells of 96-well flat bottom microtiter plates (No. 3596; Costar, Cambridge, MA) coated with fibronectin (20 μg/ml in PBS) for 24 h, after which the culture medium was replaced with ECGS free medium. Cultures were further carried out for additional 24 h with TNF-α (10 ng/ml). Various concentrations of BUC or BUC-ID were present throughout the total length of culture of 48 h. After the incubation, the wells were carefully aspirated and the cells were then fixed with 1% paraformaldehyde (PFA) for 5 min at 37 °C, followed by three times washes with PBS containing 0.05% Tween 20. After incubation with anti-VCAM-1, anti-ICAM-1, or anti-E-selectin (1 μg/ml) diluted in PBS containing 1% bovine serum albumin (Miles, Elkhart, IN) (PBS–BSA) for 1 h at 37 °C, the wells were washed with PBS-Tween 20 three times. Bound VCAM-1, ICAM-1, or E-selectin mAb were reacted with peroxidase conjugated F(ab')₂ fragments goat anti-mouse IgG (Cappel) diluted by 1:1000 in PBS–BSA for 1 h at 37 °C. Finally, the expression of VCAM-1, ICAM-1, or E-selectin was quantitated by the addition of peroxidase substrate solution containing 40 mg o-phenylenediamine and 10 μl 30% H_2O_2 in 100 ml 0.05 M citrate–phosphate buffer (pH 4.8). After incubation for 30 min at 37 °C, the reaction was stopped by addition of 5 N H_2SO_4,

and the absorbance of each well was measured at 492 nm, and the background at 630 nm was subtracted using a two-wave length microplate photometer (MTP-120, Corona Electric, Ibaraki, Japan). Within each experiment, each point was set in triplicate wells. At each point, mean ± S.D. of the triplicate wells was calculated.

2.4. Survival of HUVEC

Relative viable numbers of HUVEC was quantitated by MTT colorimetric assay after total length of cultures for 48 h. After 4 h of incubation with MTT, the reaction was stopped, and the absorbance at 570 nm was measured. Results were expressed as the mean ± S.D. of triplicate wells.

2.5. Preparation and culture of RA bone marrow CD34+ cells

Bone marrow samples were obtained from 2 patients with RA, who satisfied the American College of Rheumatology 1987 revised criteria for RA [13] and gave informed consent, during joint operations by intramedullary reaming via aspiration from a distal femoral canal prepared for implantation of an artificial femoral head. Mononuclear cells were isolated by centrifugation of heparinized bone marrow aspirates over sodium diatrizoate-Ficoll gradients (Histopaque; Sigma, St. Louis, MO). CD34+ cells were purified from the mononuclear cells by positive selection with magnetic beads (CD34 progenitor cell selection system; Dynal, Oslo, Norway). The cells thus prepared were >96% CD34+ cells and <0.5% CD19+ B cells, as previously described [14]. CD34+ cells were incubated in a 24-well microtiter plate with flat-bottomed wells (No. 3524; Costar) (10^5/well) with the presence of stem cell factor (SCF) (Pepro tech) (10 ng/ml), granulocyte macrophage-colony stimulating factor (GM-CSF) (Pepro Tech) (1 ng/ml) and tumor necrosis factor-α (TNF-α) (10 ng/ml). After 4 weeks of incubation, the culture supernatants were assayed for soluble VCAM-1.

2.6. Measurement of soluble VCAM-1

The concentrations of soluble VCAM-1 were analysed using an enzyme-linked immunosorbent assay (Cytoscreen™ Human sVCAM-1, Bio Source International, Camarillo, CA).

2.7. Statistical analysis

The significance of the effects of BUC and BUC-ID on HUVEC in each independent experiment was evaluated by Student's *t*-test. The significance of the effects of BUC-ID on the expression of VCAM-1 on HUVEC were further analysed in eight independent experiments by paired sample *t*-test.

3. Results

Initial experiments were carried out to explore the influences of pharmacologically relevant concentrations of BUC and BUC-ID on the proliferation and VCAM-1 expression of HUVEC. HUVEC were incubated with medium M199 containing heparin and 20% FCS with ECGS for 24 h, after which the culture medium was replaced with ECGS free medium. Cultures were further carried out for additional 24 h with the presence of TNF-α (10 ng/ml). Various concentrations of BUC or BUC-ID were present throughout the total length of cultures for 48 h. TNF-α increased the proliferation of HUVEC approximately twofold (OD 570: 0.144 ± 0.012 [mean ± S.D.] for medium alone vs. 0.230 ± 0.004 for TNF-α). As can be seen in Fig. 1, BUC and BUC-ID at any concentrations did not significantly influence the proliferation or viability of HUVEC. By contrast, BUC-ID, but not BUC, suppressed the expression of VCAM-1 on HUVEC stimulated with TNF-α in a dose-response manner (Fig. 2). To confirm the suppressive effect of BUC-ID on VCAM-1 expression on HUVEC, eight different experiments were carried out using different sources of HUVEC. As can be seen in Fig. 3, BUC-ID at 3.0 μg/ml (approximately 1.5×10^{-5} M) significantly suppressed the expression of VCAM-1 on TNF-α stimulated HUVEC in these eight different experiments, although the suppressive effects were relatively small (15–20% inhibition). These results indicate that at pharmacologically relevant concentrations BUC-ID suppresses the expression of VCAM-1 without affecting the proliferation of HUVEC, whereas BUC itself does not display any significant influences on the proliferation or VCAM-1 expression on HUVEC.
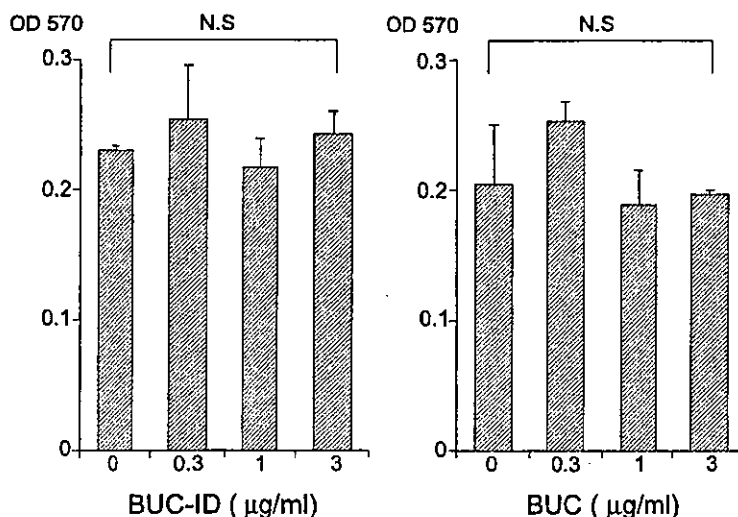
Fig. 1. Effect of BUC and BUC-ID on the proliferation of HUVEC. HUVEC (4×10^4/well) were incubated with medium M199 containing heparin and 20% FCS with endothelial cell growth supplement (ECGS) for 24 h, after which the culture medium was replaced with ECGS free medium. Cultures were further carried out for additional 24 h with TNF-α (10 ng/ml). Various concentrations of BUC or BUC-ID were present throughout the total length of cultures as indicated. After the incubation, the proliferation of HUVEC was assessed by MTT colorimetric assay. Statistical analysis was carried out by Student's t-test. Representative of eight independent experiments.

Previous studies suggested that the expression of VCAM-1 might be regulated with different mechanisms from that of ICAM-1 and E-selectin [12,15].

Next experiments therefore compared the influences of BUC and BUC-ID on the expression of VCAM-1, ICAM-1, and E-selectin. As can be seen in Table 1, at
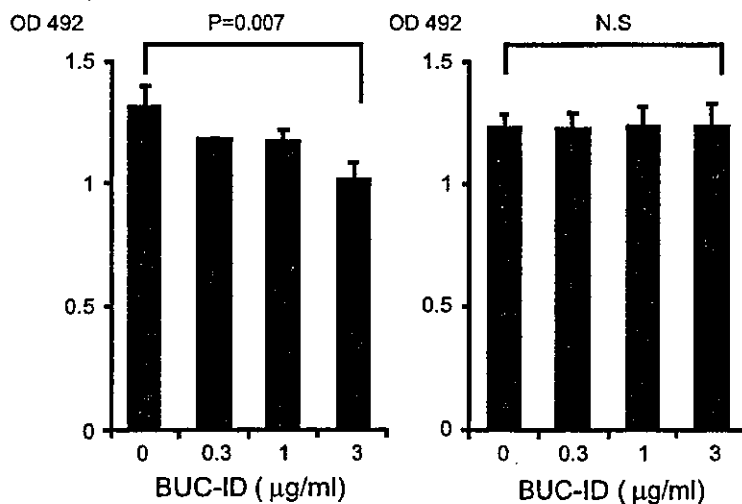

Fig. 2. Effect of BUC and BUC-ID on the expression of VCAM-1 on HUVEC. HUVEC (4×10^4/well) were incubated with medium M199 containing heparin and 20% FCS with endothelial cell growth supplement (ECGS) for 24 h, after which the culture medium was replaced with ECGS free medium. Cultures were further carried out for additional 24 h with TNF-α (10 ng/ml). Various concentrations of BUC or BUC-ID were present throughout the total length of cultures as indicated. After the incubation, the expression of VCAM-1 on HUVEC was assessed by cell ELISA. Statistical analysis was carried out by Student's t-test. Representative of eight independent experiments.
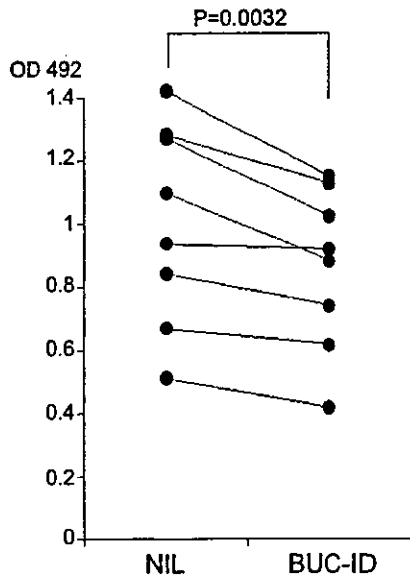
P=0.0032

OD 492

Fig. 3. Effect of BUC-ID on the expression of VCAM-1 on HUVEC. HUVEC (4×10^4/well) were incubated with medium M199 containing heparin and 20% FCS with endothelial cell growth supplement (ECGS) for 24 h, after which the culture medium was replaced with ECGS free medium. Cultures were further carried out for additional 24 h with TNF-α (10 ng/ml). BUC-ID (3 μg/ml) was present throughout the total length of cultures as indicated. After the incubation, the expression of VCAM-1 on HUVEC was assessed by cell ELISA. Statistical analysis was carried out by paired sample t-test.

a concentration of 3 μg/ml (approximately 1.5×10^{-5} M) BUC-ID, but not BUC, significantly suppressed the expression of VCAM-1 on HUVEC. By contrast, either BUC or BUC-ID did not significantly influence the expression of ICAM-1 or E-selectin on HUVEC stimulated with TNF-α. These results confirm that the expression of VCAM-1 on HUVEC is regulated by different mechanisms from that of ICAM-1 and E-selectin. Moreover, the data indicate that the suppressive effect of BUC-ID is specific for VCAM-1 in HUVEC.

A number of studies have suggested that soluble VCAM-1 (sVCAM-1) is generated through cleavage of membrane bound VCAM-1 by proteolytic enzyme [16,17]. It was thus possible that the down-regulation of the expression of VCAM-1 on HUVEC by BUC-ID might be a result of increased cleavages of VCAM-1. To test this possibility, the next experiment examined the effects of BUC-ID on

the production of sVCAM-1 by HUVEC stimulated with TNF-α. As shown in Fig. 4, BUC-ID also suppressed the production of sVCAM-1 by HUVEC stimulated with TNF-α. Of note, the inhibitory effects of BUC-ID on the production of sVCAM-1 were much more striking than those on the surface expression of VCAM-1. The data therefore indicate that BUC-ID inhibits the synthesis of VCAM-1, leading to the decreased expression of VCAM-1 on the surface of HUVEC as well as the decreased release of sVCAM-1.

It has been recently shown that RA bone marrow CD34+ cells have enhanced capacities to induce activation of endothelial cells to express adhesion molecules, such as VCAM-1 [18]. Final experiments were thus designed to examine the effects of BUC-ID and BUC on the production of sVCAM-1 by RA bone marrow CD34+ cells stimulated with SCF, GM-CSF and TNF-α for 4 weeks. As shown in Fig. 5, BUC-ID (0.66 μg/ml) as well as BUC (3 μg/ml) suppressed the production of sVCAM-1. It should be noted that BUC-ID at the lower concentration (0.66 μg/ml) displayed more striking suppressive effects than BUC at the higher concentration (3 μg/ml). The results strongly suggest that BUC-ID might suppress the activation of endothelial cells in RA the way it works in HUVEC.

Table 1
Differential effects of BUC-ID on the TNF-α induced expression of various adhesion molecules on HUVEC

Inhibitors	Expression of adhesion molecules (OD492)		
	VCAM-1	ICAM-1	E-selectin
Nil	1.232 ± 0.046	2.263 ± 0.033	0.471 ± 0.040
BUC	1.238 ± 0.087	2.399 ± 0.044	0.511 ± 0.016
BUC-ID	$1.023 \pm 0.067^*$	2.091 ± 0.152	0.487 ± 0.011

HUVEC (4×10^4/well) were incubated with medium M199 containing heparin and 20% FCS with endothelial cell growth supplement (ECGS) for 24 h, after which the culture medium was replaced with ECGS free medium. HUVEC were stimulated with TNF-α (10 ng/ml) for 3 (E-selection) or 24 h (VCAM-1, ICAM-1). Either BUC (3.0 μg/ml) or BUC-ID (3.0 μg/ml) was present throughout the total length of cultures. After the incubation, the expression of various adhesion molecules was assessed by cell ELISA. Data are expressed mean ± S.D. of triplicate determinations. Statistical analysis was carried out by Student's t-test. Representative of two independent experiments.

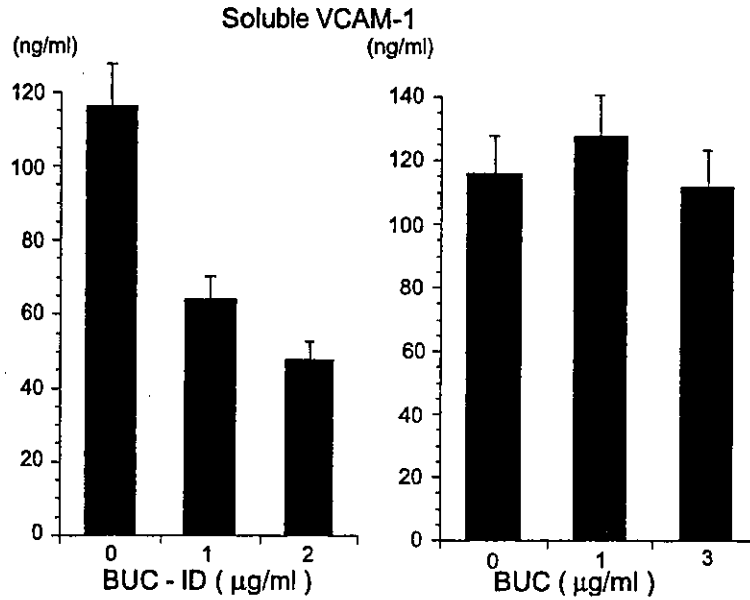* Significantly suppressed compared with medium alone (Nil) at $p < 0.05$.

Fig. 4. Effect of BUC and BUC-ID on the production of soluble VCAM-1 by HUVEC. HUVEC (4 × 10⁴/well) were incubated with medium M199 containing heparin and 20% FCS with endothelial cell growth supplement (ECGS) for 24 h, after which the culture medium was replaced with ECGS free medium. Cultures were further carried out for additional 24 h with TNF-α (10 ng/ml). Various concentrations of BUC or BUC-ID were present throughout the total length of cultures as indicated. After the incubation, the culture supernatants were harvested and the concentration of soluble VCAM-1 was measured by ELISA. Representative of two independent experiments.
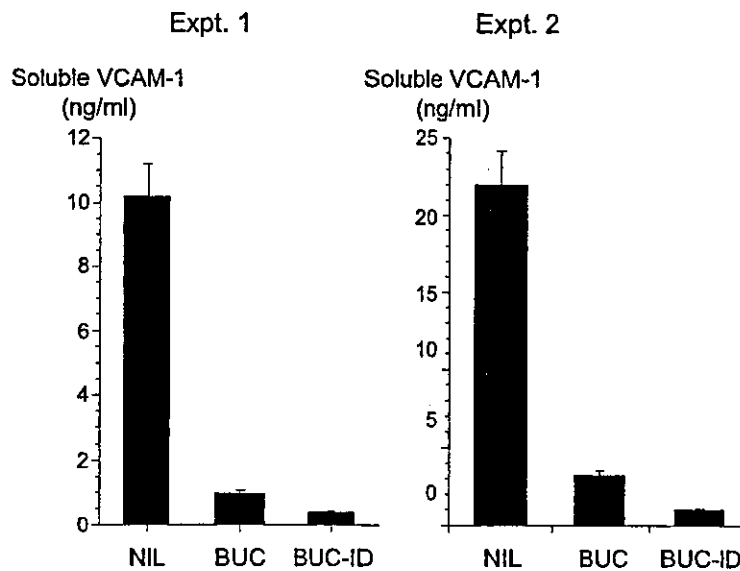

Fig. 5. Effect of BUC and BUC-ID on the production of soluble VCAM-1 by RA bone marrow CD34+ cells. RA bone marrow CD34+ cells were cultured in the presence of SCF (10 ng/ml), GM-CSF (1 ng/ml) and TNF-α (10 ng/ml) with or without BUC (3 μg/ml) or BUC-ID (0.66 μg/ml). After 4 weeks of incubation, the culture supernatants were harvested and the concentration of soluble VCAM-1 was measure by ELISA.

4. Discussion

The results in the present study have demonstrated that BUC-ID, an intramolecular disulfide form of BUC, inhibits the expression of VCAM-1 on HUVEC at its pharmacologically relevant concentrations, although the suppressive effects were relatively small. Thus, BUC-ID significantly suppressed the expression of VCAM-1 on HUVEC at the concentration of 3 μg/ml. Although the plasma concentrations of BUC-ID and BUC have been shown to reach 2.0 μg/ml in normal individuals [19], no data are available in RA patients. Since the plasma concentrations of a similar compound D-penicillamine have been shown to be 1–3 μg/ml in RA patients [20], it is conceivable that BUC-ID might reach 3 μg/ml in RA patients. Since BUC-ID did not suppress the proliferative response of HUVEC, it is clear that its inhibitory effects on VCAM-1 expression are not due to suppression of cell growth or induction of apoptosis. In addition, BUC-ID also suppressed the production of sVCAM-1 by HUVEC, obviating the possibility that BUC-ID might promote the proteolytic cleavage of VCAM-1 expressed on the surface of HUVEC. The data therefore strongly suggest that BUC-ID might inhibit de novo synthesis of VCAM-1 in HUVEC. Of interest, BUC itself did not display the capacity to inhibit the expression of VCAM-1 on HUVEC. In this regard, the differential action of BUC and BUC-ID on HUVEC is comparable to that on human B cells. Thus, BUC-ID suppressed the production of IgM much more effectively than BUC at pharmacologically relevant concentrations [4]. It is therefore suggested that BUC-ID might play more important roles than BUC itself in the expression of antirheumatic effects of BUC in RA patients.

The hallmarks of the pathological changes in the RA synovium include hyperplasia of synovial lining cells and follicle-like aggregation of lymphocytes and plasma cells [6]. A number of studies have demonstrated that angiogenesis as well as activation of endothelial cells plays a crucial role in recruiting lymphocytes and monocyte-lineage cells [7–9]. In fact, therapeutic intervention of angiogenesis, such as angiostatin, has been shown to inhibit the development of murine type II collagen arthritis [21]. Moreover, several disease-modifying anti-rheumatic drugs have been found to suppress the production of angiogenic factors by synovial cells [10,22]. Thus, BUC suppressed the pro-

duction of VEGF by cultured synovial cells as well as decreased the serum VEGF concentrations in RA patients [22]. In addition to the inhibitory effects of BUC in VEGF production, the results in the current studies have demonstrated that BUC-ID suppresses VCAM-1 expression on HUVEC. It is therefore suggested that these effects of BUC and BUC-ID on angiogenesis and endothelial cell activation might also account at least in part for the antirheumatic effects of BUC, in addition to their effects on immunocompetent cells, such as B cells and T cells [4,5].

Of note, the inhibitory effects of BUC-ID on the secretion of sVCAM-1 by HUVEC were much more striking than those on the surface expression of VCAM-1. It has been recently disclosed that tumor necrosis factor-α-converting enzyme (TACE) plays a pivotal role in the shedding of VCAM-1 [23]. It is therefore possible that BUC-ID might also inhibit the activity and/or the expression of TACE. Since previous studies suggested that TACE might also be involved in the pathogenesis of RA synovitis [24], further studies to delineate the effects of BUC-ID on TACE would be important for a complete understanding of the mechanism of action of BUC in RA.

The results in the present study also disclosed that BUC-ID also suppressed the production of sVCAM-1 by RA bone marrow CD34+ cells stimulated with SCF, GM-CSF and TNF-α, much more effectively than BUC. It has been shown that bone marrow CD34+ progenitor cells contain progenitors of endothelial cells that respond to SCF and GM-CSF [25]. It is therefore likely that BUC-ID might suppress the activation of endothelial cells in RA the way it works in HUVEC, although it is also possible that BUC-ID might inhibit the development of endothelial cells from RA bone marrow CD34+ cells.

Acknowledgements

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Enhanced Generation of Endothelial Cells From CD34+ Cells of the Bone Marrow in Rheumatoid Arthritis

Possible Role in Synovial Neovascularization

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Objective. To examine the capacity of bone marrow CD34+ cells to generate endothelial cells, in order to assess the role of bone marrow in neovascularization in the synovium of rheumatoid arthritis (RA).

Methods. CD34+ cells purified from the bone marrow of 13 patients with active RA and 9 control subjects (7 osteoarthritis [OA] patients and 2 healthy individuals) were cultured in the presence of stem cell factor (10 ng/ml) and granulocyte–macrophage colony-stimulating factor (1 ng/ml). After 18 days of incubation, the generation of endothelial cells was assessed by flow cytometry. The generation of endothelial cells was compared with the degree of vascularization in the synovial tissues and with the microvessel densities in the synovium, as determined by microscopy. The expression of vascular endothelial growth factor receptor 2/kinase insert domain receptor (KDR) messenger RNA (mRNA) in CD34+ cells was examined by quantitative reverse transcription–polymerase chain reaction.

Results. The generation of CD14+ cells from bone marrow–derived CD34+ cells from RA patients was comparable to that from control subjects. However, the generation of von Willebrand factor (vWF)–positive cells and CD31+/vWF+ cells from RA bone marrow–derived CD34+ cells was significantly higher than that from control subjects ($P = 0.004$ and $P = 0.030$, respectively). The generation of vWF+ cells from bone marrow CD34+ cells correlated significantly with the microvessel densities in the synovial tissues ($r = 0.569$, $P = 0.021$). Finally, RA bone marrow CD34+ cells expressed KDR mRNA at higher levels than OA bone marrow CD34+ cells.

Conclusion. These results indicate that RA bone marrow CD34+ cells have enhanced capacities to differentiate into endothelial cells in relation to synovial vascularization. The data therefore suggest that bone marrow CD34+ cells might contribute to synovial neovascularization by supplying endothelial precursor cells and, thus, play an important role in the pathogenesis of RA.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by hyperplasia of synovial lining cells (1). Synovial lining cells consist of type A (macrophage-like) synoviocytes and type B (fibroblast-like) synoviocytes. Recent studies have suggested that type A synoviocytes are derived from monocyte precursors in the bone marrow (2). Accordingly, it has been shown that the spontaneous generation of CD14+ cells from bone marrow–derived CD14– progenitor cells is accelerated in RA, resulting in the facilitated entry of such CD14+ cells into the synovium (3). On the other hand, type B synoviocytes have a morphologic appearance of fibroblasts as well as the capacity to produce and secrete a variety of factors, including proteoglycans, cytokines, arachidonic acid metabolites, and matrix met-

alloproteinases (MMPs), that lead to the destruction of joints (4). Whereas type B synoviocytes are thought to arise from the sublining tissue or other support structures of the joint (4), recent studies have suggested that they are also derived from bone marrow progenitor cells (5). Increasing attention has therefore been given to the role of the bone marrow in the pathogenesis of RA (6).

In the RA joint, the massive proliferating synovium forms an invading tissue called pannus, which results in the destruction of cartilage and bone. A number of studies have shown that persistent neovascularization is a crucial support to the continuous proliferation of the synovium, through delivery of nutrients and recruitment of inflammatory cells into the synovium (7,8). It was a long-held belief that vessels in the embryo developed from endothelial progenitors (vasculogenesis), whereas spouting of vessels in the adult resulted only from division of differentiated endothelial cells (angiogenesis) (9). Thus, the neovascularization in RA synovium has been attributed to angiogenesis, a process characterized by spouting of new capillaries from preexisting blood vessels (10).

Asahara et al (11), however, isolated endothelial progenitor cells from adult human peripheral blood using magnetic bead selection of CD34+ hematopoietic cells, and thus demonstrated that human peripheral blood CD34+ cells differentiated in vitro into endothelial cells, which expressed endothelial markers, including CD31. In addition, those investigators found that human CD34+ cells were incorporated into neovascularized hind limb ischemic sites in animal models (11). Since the time these observations were reported, it has also been found that endothelial progenitor cells capable of contributing to capillary formation can be derived from the bone marrow, possibly playing a role in the de novo formation of capillaries without preexisting blood vessels (12–14). Thus, the accumulating evidence has suggested that bone marrow–derived endothelial cells might be involved in several disorders characterized by excessive angiogenesis, such as myocardial infarction (15). However, the role of bone marrow in RA synovial neovascularization has not been explored.

It has been demonstrated that early endothelial progenitor cells in bone marrow express CD34, CD133, and vascular endothelial growth factor receptor 2 (VEGFR-2)/kinase insert domain receptor (KDR) (15). In general, early endothelial progenitor cells in the bone marrow are positive for CD34/CD133/VEGFR-2, whereas circulating endothelial progenitor cells are positive for CD34/VEGFR-2/CD31, negative for CD133,

and are beginning to express von Willebrand factor (vWF) (15). Thus, it appears that vWF is expressed on fully matured endothelial cells.

The current studies were undertaken to explore whether CD34+ cells derived from the bone marrow of RA patients might have an enhanced capacity to generate endothelial cells so that we could assess the role of the bone marrow in the neovascularization of RA synovium. The results clearly indicate that bone marrow–derived CD34+ cells from RA patients differentiate into vWF+ endothelial cells upon stimulation with stem cell factor (SCF) and granulocyte–macrophage colony-stimulating factor (GM-CSF) much more effectively than do those from control subjects. The data therefore suggest that bone marrow CD34+ cells might play a role in the synovial hyperplasia in RA through mobilization of endothelial progenitor cells into the synovium, where angiogenesis is activated.

MATERIALS AND METHODS

Patients and samples. Bone marrow samples from 13 RA patients (1 man and 12 women; mean age 58.2 years [age range 45–72 years]) were obtained during joint operation through aspiration from the iliac crest. All RA patients met the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria (16). As controls, bone marrow samples were similarly obtained from 7 patients with osteoarthritis (OA) (7 women; mean age 70.6 years [age range 67–74]). All study patients gave their informed consent for study. In addition, bone marrow–derived CD34+ cells from 2 healthy individuals (2 men; ages 27 years and 24 years) were purchased from BioWhittaker (Walkersville, MD). Synovial tissues were also obtained from 10 of the RA patients and 6 of the OA patients during the same joint operation.

A second group of bone marrow samples was obtained from an additional 10 RA patients (3 men and 7 women; mean age 62.6 years) and an additional 4 OA patients (2 men and 2 women; mean age 72.6 years). These bone marrow samples were used in analyses of the expression of KDR messenger RNA (mRNA). These patients also gave their informed consent for study. In addition, samples from 7 of the RA patients and 6 of the OA patients from the first group described above were included in the study of KDR mRNA expression.

Culture medium and reagents. RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), L-glutamine (0.3 mg/ml), and 10% fetal bovine serum (Life Technologies) was used for all cultures. Recombinant human GM-CSF and SCF were purchased from PeproTech (London, UK).

Preparation and culture of bone marrow–derived CD34+ cells. Mononuclear cells were isolated by centrifugation of heparinized bone marrow aspirates over sodium

Figure 1. Histologic features (A) and immunohistochemistry of CD31 cells (B) in the synovium of patients with osteoarthritis (OA) and rheumatoid arthritis (RA). Four representative samples of synovium from OA (a and b) and RA (c and d) patients are presented, showing trace (a), mild (b), moderate (c), and strong (d) neovascularization. H&E = hematoxylin and eosin. (Original magnification × 25.)

diatrizoate-Ficoll gradients (Histopaque; Sigma, St. Louis, MO). CD34+ cells were purified from the mononuclear cells through positive selection using magnetic beads (Dynal CD34 progenitor cell selection system; Dynal, Oslo, Norway). CD34+ cells thus prepared were ~95% CD34+ cells and <0.5% CD19+ B cells, as previously described (5).

CD34+ cells were incubated in a 24-well microtiter plate with flat-bottomed wells (no. 3524; Costar, Cambridge, MA) at a density of 1.0×10^5/well in the presence of SCF (10 ng/ml) and GM-CSF (1 ng/ml). After 18 days of incubation, the cells were stained with various antibodies and analyzed by flow cytometry.

Immunofluorescence staining and analysis. Cultured CD34+ cells were stained with saturating concentrations of antibodies, including fluorescein isothiocyanate (FITC)–conjugated anti–HLA–DR monoclonal antibody (mAb) (mouse IgG2b; Immunotech, Marseilles, France), FITC-conjugated sheep anti-vWF IgG (Cosmo Bio, Tokyo, Japan), phycoerythrin (PE)–conjugated anti-CD14 mAb (mouse IgG2a; Immunotech), PE-conjugated anti-CD31 mAb (mouse IgG1; Immunotech), PE-conjugated murine IgG1 and IgG2a control mAb or FITC-conjugated murine IgG2b control mAb, which were raised against *Aspergillus niger* glucose oxide, an enzyme that is neither present nor inducible in mammalian tissues (Dako, Glostrup, Denmark), or FITC-conjugated control sheep IgG purified from normal sheep serum (Rockland, Gilbertsville, PA).

Briefly, the cells were washed with 2% normal human serum in phosphate buffered saline (PBS), pH 7.2, and 0.1% sodium azide (staining buffer), and the cells were stained with saturating concentrations of a variety of antibodies at 4°C for 30 minutes. The cells were then washed 3 times with staining buffer and fixed with 1% paraformaldehyde in PBS for at least 5 minutes at room temperature. Cells were analyzed using an

Epics XL flow cytometer (Coulter, Hialeah, FL) equipped with an argon-ion laser at 488 nm. A combination of low-angle and 90° light scatter measurements (forward scatter versus side scatter) was used to identify bone marrow cells. The percentages of cells that stained positive for each mAb were determined by integration of cells above a specified fluorescence channel, which was calculated in relation to an isotype-matched control mAb.

Synovial histopathology and determination of microvessel densities. Synovial tissues were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. To visualize endothelial cells in the synovium, the paraffin-embedded sections were also stained with murine anti-CD31 mAb (clone JC/70A; Dako) and then developed using a Dako Envision kit, which includes horseradish peroxidase and diaminobenzidine. The degree of neovascularization was analyzed under light microscopy and scored as 0 (trace), 1 (mild), 2 (moderate), or 3 (strong) (Figures 1A and B). Grades were assigned by 2 independent observers (SH and TY) who had no knowledge of the diagnosis of the patients from whom the tissues had been obtained. When grades differed (2 of 16 cases), the synovium was reexamined, and a consensus was reached.

Sections were photographed with an Olympus DP11 digital camera (Olympus, Tokyo, Japan), and the CD31+ microvessel densities were determined by counting the vascular structures with a clearly defined lumen or linear shape as seen on the photographs. The final microvessel density was calculated as the mean score of the 3 1-mm² fields with the highest individual scores (17).

Measurement of cytokines in the culture supernatants. Concentrations of tumor necrosis factor α (TNFα) and vascular endothelial growth factor (VEGF) in the culture supernatants were measured by enzyme-linked immunosorbent assay

(ELISA) using a human TNFα ELISA kit (PeproTech) and a human VEGF immunoassay kit (BioSource International, Camarillo, CA).

RNA isolation and real-time quantitative polymerase chain reaction. Total RNA was isolated from purified bone marrow CD34+ cells using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Complementary DNA (cDNA) samples were prepared from 1 μg of total RNA using the SuperScript reverse transcriptase preamplification system (Life Technologies) with oligo(dT) primers and were subjected to PCR. Real-time quantitative PCR was performed using the LightCycler rapid thermal cycler system (Roche Diagnostics, Lewes, UK) with primer sets for VEGFR-2/KDR or β-actin (Nihon Gene Research Laboratories, Sendai, Japan) and LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics).

The primer sequences were as follows: for KDR, 5'-CAGACGGACAGTGGTATGGT-3' (forward) and 5'-GCCTTCAGATGCCACAGACT-3' (reverse); and for β-actin, 5'-GCAAAGACCTGTACGCCAAC-3' (forward) and 5'-CTAGAAGCATTTGCGGTGGA-3' (reverse). The PCR reaction conditions were as follows: denaturing at 95°C for 10 minutes for 1 cycle, followed by 40 cycles of denaturing at 95°C for 10

seconds, annealing at 62°C for 10 seconds, and extension at 72°C for 5 seconds (KDR) or 10 seconds (β-actin). Quantitative analysis was performed using LightCycler software version 3.5. All results for KDR were calibrated to the copy number of β-actin from each cDNA sample.

RESULTS

Synovial histopathologic features in RA. A number of studies have confirmed that the microscopic appearance of RA synovial tissue is variable (18). Consistent with those studies, Figures 1A and B show representative patterns of neovascularization seen in RA synovium in this study. It appears that the overall degree of neovascularization is correlated with the degree of exudation, cellular infiltration, and granulation tissue development, which are characteristic features of RA synovium (18). Thus, synovium with marked granulation and cellular infiltration showed the maximal degree of neovascularization (Figures 1A and B part
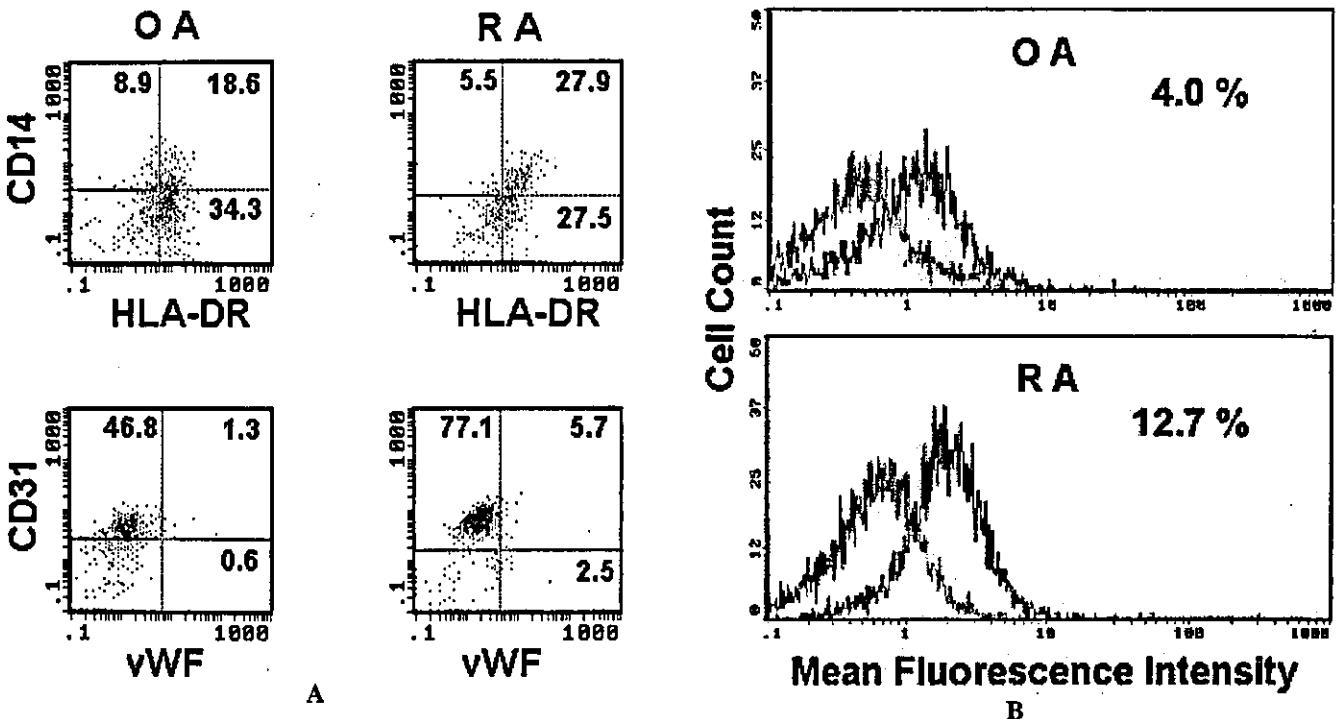

Figure 2. Representative 2-color and single-color flow cytometric analyses of the phenotypes of CD34+ cells stimulated with stem cell factor (SCF) and granulocyte–macrophage colony-stimulating factor (GM-CSF). CD34+ cells from the bone marrow of a rheumatoid arthritis (RA) and an osteoarthritis (OA) patient were stimulated for 18 days with SCF and GM-CSF, stained with phycoerythrin (PE)–conjugated anti-CD14 and fluorescein isothiocyanate (FITC)–conjugated anti–HLA–DR or with PE-conjugated anti-CD31 and FITC-conjugated von Willebrand factor (vWF), and analyzed by flow cytometry. A, The quadrant boundaries were determined by analysis of isotype-matched control cells. Values shown are percentages of cells. B, Single-color analysis of the expression of vWF (left histogram). Right histogram shows control staining. Percentages shown are for vWF+ cells. Figure 2B can be viewed in color in the online issue, which is available at http://www.arthritisrheum.org.
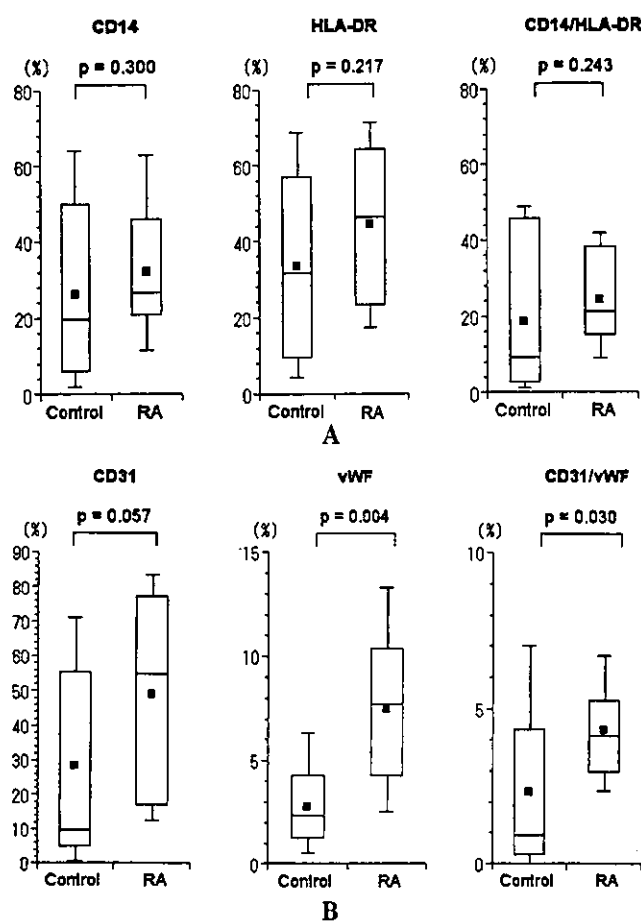
Figure 3. Phenotypic features of bone marrow–derived CD34+ cells stimulated with SCF and GM-CSF. Bone marrow CD34+ cells from 13 RA patients and 9 control subjects were stimulated for 18 days with SCF and GM-CSF, stained with A, PE-conjugated anti-CD14 and FITC-conjugated anti–HLA-DR or B, PE-conjugated anti-CD31 and FITC-conjugated anti-vWF, and analyzed by flow cytometry. Data are shown as box plots. Horizontal lines constituting the top, middle, and bottom of the boxes show the 75th, 50th, and 25th percentiles, respectively. Lines outside the boxes show the 90th and 10th percentiles. Solid squares inside the boxes show the mean. P values were determined by Mann-Whitney U test. See Figure 2 for definitions.

d), whereas the synovium with trace amounts of exudation and cellular infiltration lacked neovascularization. The results therefore suggest that neovascularization might play a role in development of inflamed synovium in RA.

Generation of vWF+ cells from bone marrow–derived CD34+ cells. After stimulation of bone marrow CD34+ cells (1.0 × 10^5) with SCF and GM-CSF for 18 days, the mean ± SD number of recovered cells was 3.85 ± 2.97 × 10^5 for RA patients and 3.79 ± 3.18 ×

10^5 for control subjects ($P = 0.6401$ by Mann-Whitney U test). Figures 2A and B show, respectively, the representative dual-parameter 4-quadrant scattergrams and single-color histograms of the bone marrow CD34+ cells stimulated with SCF and GM-CSF for 18 days.

A similar percentage of cultured CD34+ cells from the RA patient and the OA control patient expressed CD14 and HLA–DR. Although in Figure 2A, bone marrow CD34+ cells from the RA patient generated higher percentages of CD14+/HLA–DR+ cells, there was no significant difference in the percentages of CD14+ cells and CD14+/HLA–DR+ cells generated by bone marrow CD34+ cells from the 9 control subjects and the 13 RA patients (Figure 3A). In contrast, bone marrow CD34+ cells from the RA patient gave rise to higher numbers of vWF+ cells and CD31+/vWF+ cells than did those from the OA control patient (Figures 2A
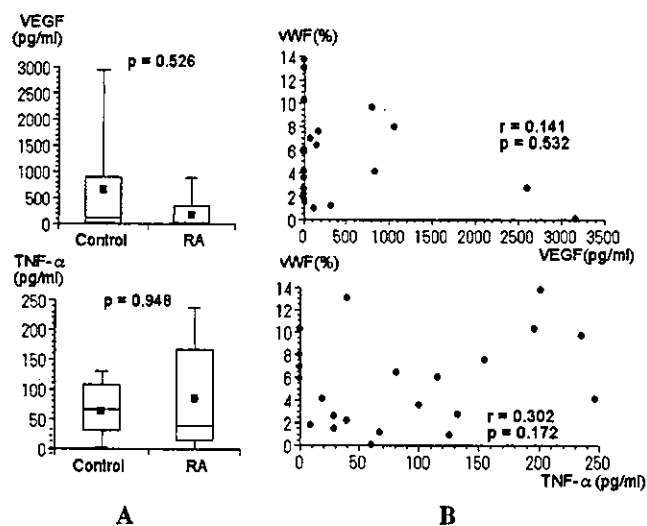

Figure 4. Production of vascular endothelial growth factor (VEGF) and tumor necrosis factor α (TNFα) by bone marrow–derived CD34+ cells and correlation with the generation of von Willebrand factor (vWF)–positive cells. Bone marrow CD34+ cells from 13 rheumatoid arthritis (RA) patients and 9 control subjects were stimulated for 18 days with stem cell factor and granulocyte–macrophage colony-stimulating factor. Cells were analyzed for vWF expression by flow cytometry; supernatants were assayed for VEGF and TNFα by enzyme-linked immunosorbent assay. A, Data are shown as box plots. Horizontal lines constituting the top, middle, and bottom of the boxes show the 75th, 50th, and 25th percentiles, respectively. Lines outside the boxes show the 90th and 10th percentiles. Solid squares inside the boxes show the mean. P values were determined by Mann-Whitney U test. B, Correlations between the generation of vWF+ cells and the production of each cytokine was evaluated by simple regression analysis.

and B). Accordingly, bone marrow CD34+ cells from the 13 RA patients generated significantly higher numbers of vWF+ cells as well as CD31+/vWF+ cells than did those from the 9 control subjects, although there was no significant difference in the number of CD31+ cells (Figure 3B). These results indicate that upon stimulation with SCF and GM-CSF, bone marrow CD34+ cells from RA patients have enhanced capacities to generate endothelial cells compared with control subjects, whereas the capacities of RA bone marrow CD34+ cells to give rise to CD14+ monocyte-lineage cells were comparable to those of the control subjects.

Relationship between the generation of vWF+ cells and the production of VEGF and TNFα. It was possible that the enhanced generation of vWF+ cells from RA bone marrow–derived CD34+ cells might be a result of the increased production of angiogenic cytokines. In fact, it has been reported that VEGF (19,20) and TNFα (21–24) play a major role in regulating neovascularization in RA. Our next experiments therefore examined the capacity of bone marrow CD34+ cells stimulated with SCF plus GM-CSF to produce VEGF and TNFα. As can be seen in Figure 4, there were no significant differences in the production of VEGF and TNFα by RA bone marrow CD34+ cells and control bone marrow CD34+ cells. In addition, the generation of vWF+ cells was not significantly correlated with the production of VEGF or TNFα. The results therefore suggest that the enhanced generation of vWF+ cells from RA bone marrow CD34+ cells might not be due to the increased production of angiogenic cytokines but, more likely, it may be due to the intrinsic abnormalities of the bone marrow CD34+ cells.

Relationship between the capacity of bone marrow–derived CD34+ cells to generate endothelial cells and the vascularization of the synovium. To further confirm the role of the bone marrow in synovial neovascularization, we next examined the relationship between the capacity of bone marrow CD34+ cells to generate vWF+ cells and the degree of vascularization in the synovium in synovium samples obtained from 10 of the RA patients and 6 of the OA patients on the same day as the bone marrow samples. The degree of vascularization in the synovium was analyzed under light microscopy and scored as described in Materials and Methods. The degree of synovial vascularization in the RA patients was significantly elevated compared with that in the OA patients. In addition, the capacity of bone marrow CD34+ cells to generate vWF+ cells was significantly

correlated with the degree of vascularization in the synovium in these 16 patients (data not shown).

To evaluate the synovial vascularization in a more objective manner, we calculated the CD31+ microvessel densities in the synovium. As shown in Figure 5, the synovial microvessel densities were significantly higher in RA patients than in OA patients. Moreover, the microvessel densities were also significantly correlated with the generation of vWF+ cells from bone marrow–derived CD34+ cells in the 16 patients with OA or RA. The results therefore suggest that vasculogenesis occurring through mobilization of endothelial progenitor cells from the bone marrow might be involved, at least in part, in the synovial neovascularization, and may thus play a significant role in the pathogenesis of RA.

Expression of mRNA for VEGFR-2/KDR in bone marrow–derived CD34+ cells. Recent studies have revealed that the vascular activation by VEGF/KDR was significantly higher in RA than in OA, although activation of the hypoxia inducing factor α (HIFα) pathway was comparable in RA and OA (25). It was therefore possible that there might be differences in the activation
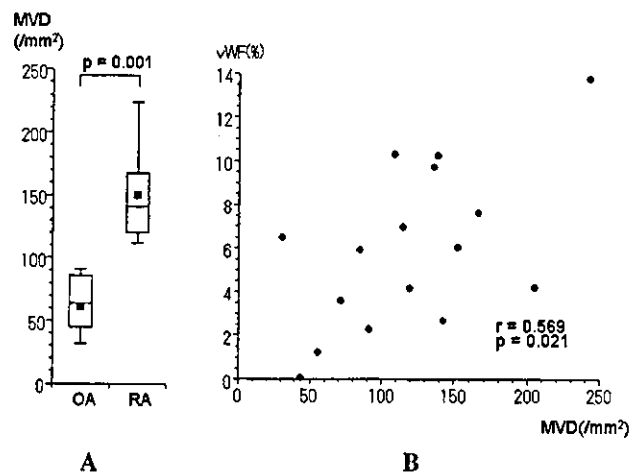

Figure 5. Correlation between synovial microvessel densities (MVD) and the generation of von Willebrand factor (vWF)–positive cells from bone marrow–derived CD34+ cells. Synovial microvessel densities were compared between 10 rheumatoid arthritis (RA) patients and 6 osteoarthritis (OA) patients. A, Data are shown as box plots. Horizontal lines constituting the top, middle, and bottom of the boxes show the 75th, 50th, and 25th percentiles, respectively. Lines outside the boxes show the 90th and 10th percentiles. Solid squares inside the boxes show the mean. P values were determined by Mann-Whitney U test. B, Correlations between the microvessel densities and the generation of vWF+ cells from bone marrow CD34+ cells in the 16 patients was determined by simple regression analysis.
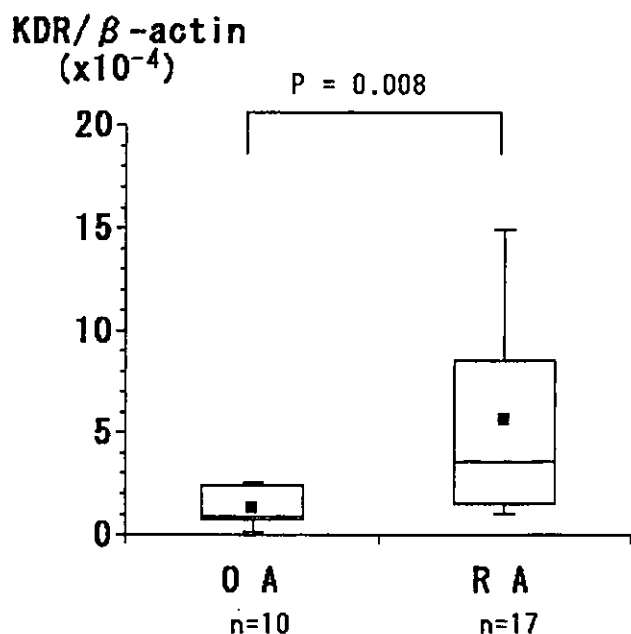
KDR/β-actin
(x10^{-4})

Figure 6. The expression of vascular endothelial growth factor receptor 2 (VEGFR-2)/kinase insert domain receptor (KDR) mRNA in bone marrow–derived CD34+ cells. RNA was extracted from bone marrow CD34+ cells obtained from rheumatoid arthritis (RA) and osteoarthritis (OA) patients and subjected to quantitative reverse transcription–polymerase chain reaction for VEGFR-2/KDR. The copy numbers for VEGFR-2/KDR mRNA were calibrated to those for β-actin. Data are shown as box plots. Horizontal lines constituting the top, middle, and bottom of the boxes show the 75th, 50th, and 25th percentiles, respectively. Lines outside the boxes show the 90th and 10th percentiles. Solid squares inside the boxes show the mean. P values were determined by Mann-Whitney U test.

of the VEGF/KDR pathway in bone marrow CD34+ cells. To explore this possibility, our final experiments examined the expression of KDR mRNA in bone marrow CD34+ cells as measured by quantitative reverse transcription–PCR. As can be seen in Figure 6, the expression of VEGFR-2/KDR mRNA in bone marrow CD34+ cells from 17 patients with RA was significantly higher than that in bone marrow CD34+ cells from 10 patients with OA. The results therefore suggest that up-regulation of KDR mRNA in bone marrow CD34+ cells in RA might result in their enhanced capacity to generate endothelial cells.

DISCUSSION

A number of studies have indicated that neovascularization is crucial to the synovial hyperplasia of RA (7,8). Postnatal neovascularization has been attributed

to so-called angiogenesis, a process characterized by the sprouting of new capillaries from preexisting blood vessels (10). Thus, it has been shown that the expression of angiogenic factors, such as VEGF and basic fibroblast growth factor in synovial lining cells and stromal cells, is increased in RA synovium and plays a pivotal role in the angiogenesis (19,20,23). It is noteworthy that recent studies have demonstrated that endothelial progenitor cells of bone marrow origin play a significant role in the de novo formation of capillaries without preexisting blood vessels, so-called vasculogenesis (11–14). Moreover, bone marrow–derived endothelial precursor cells have been shown to home to neovascularized hind limb ischemic sites in animal models (11). Results of the current studies have shown that the generation of vWF+ endothelial cells from bone marrow CD34+ cells is up-regulated in RA. The data therefore suggest that mobilization of endothelial cells from bone marrow might also be enhanced and be involved in neovascularization of the RA synovium. It is thus likely that bone marrow–derived endothelial precursor cells might be homing to the synovium, where angiogenesis is enhanced (7,8).

It has been shown that bone marrow–derived endothelial progenitor cells make a significant contribution to angiogenic growth factor–induced neovascularization that may account for up to 26% of all endothelial cells (26,27). It is therefore likely that the enhanced capacity of bone marrow CD34+ cells to generate vWF+ cells might also play a critical role in the synovial neovascularization in RA. In fact, we found that the degree of synovial vascularization as well as the microvessel densities in RA synovium were much higher than those in OA synovium, findings that are consistent with those of a previous study (28). More important, the generation of vWF+ cells from bone marrow–derived CD34+ cells was significantly correlated with the degree of synovial vascularization as well as with the microvessel densities in arthritis patients. The data therefore raise the possibility that the mobilization of endothelial progenitor cells from bone marrow might also contribute to the enhanced synovial neovascularization in RA, although a direct role for these bone marrow–derived cells in synovial neovascularization remains to be elucidated. Further studies to explore in detail the capacity of endothelial progenitor cells generated from bone marrow CD34+ cells to undergo angiogenesis would be important.

Previous studies have shown that hematopoietic cytokines, such as SCF and GM-CSF, have potent

effects on endothelial cells and facilitate angiogenesis (29). In the current studies, we demonstrated that SCF and GM-CSF also induce the generation of endothelial cells from bone marrow CD34+ cells and, thus, participate in vasculogenesis. The mechanism of the enhanced generation of endothelial cells from RA bone marrow CD34+ cells stimulated with SCF and GM-CSF is still unclear. It is possible that the production of VEGF, presumably by CD14+ cells induced from CD34+ cells, might be enhanced in cultures of RA bone marrow–derived CD34+ cells. However, there were no significant differences in the production of VEGF between RA patients and control subjects. Moreover, the generation of vWF+ cells was not significantly correlated with the production of VEGF. It is therefore unlikely that the enhanced generation of vWF+ cells from RA bone marrow CD34+ cells might result from the up-regulation of VEGF production.

TNFα plays a crucial role in regulating not only inflammation, but also neovascularization in RA synovium (21–24). Anti-TNFα treatment in RA patients has been found to inhibit vascularity in the synovium (30,31). The results of the current studies revealed that significant amounts of TNFα were produced in cultures of bone marrow CD34+ cells. However, there were no significant differences in the production of TNFα by bone marrow CD34+ cells from RA patients and control subjects, nor was the generation of vWF+ cells significantly correlated with the production of TNFα. Therefore, the enhanced generation of vWF+ cells might not be accounted for by the increased production of TNFα, although it is still possible that up-regulation of the production of angiogenic factors other than VEGF and TNFα might be involved in the enhanced generation of vWF+ cells from RA bone marrow CD34+ cells. Alternatively, it is also possible that the reactivity of RA bone marrow CD34+ cells to various cytokines might be different from that of control bone marrow CD34+ cells. In this regard, previous studies have shown that RA bone marrow CD34+ cells have abnormal capacities to respond to TNFα and differentiate into fibroblast-like cells producing MMP-1 (5).

Neovascularization of the synovium is not unique to RA. It has also been observed in OA synovium and has been shown to play an important role in the development of new cartilage and mineralization (25,32,33). Of note, recent studies have revealed that levels of expression of the angiogenic factors VEGF and platelet-derived endothelial cell growth factor are increased in RA as well as in OA, relative to normal subjects,

whereas the presence of an activated synovial vasculature was high only in RA (25). Moreover, the vascular activation by VEGF/KDR was significantly lower in OA than in RA patients, although the activation of the HIFα pathway was comparable in OA and RA patients (25). These observations suggest the presence of intrinsic abnormalities in synovial endothelial cells in RA patients. Of note, in the present study, RA bone marrow CD34+ cells displayed a higher capacity to generate vWF+ endothelial cells than did OA bone marrow CD34+ cells. Moreover, the expression of VEGFR-2/KDR mRNA in RA bone marrow CD34+ cells was significantly higher than that in OA bone marrow CD34+ cells. It is therefore likely that the differences in VEGF/KDR vascular activation at the level of bone marrow CD34+ cells between RA and OA patients might result in differences in their capacity to generate vWF+ cells, since signaling through the KDR plays a crucial role in the generation of endothelial cells (17,19). Further studies to delineate the precise sequelae of the up-regulation of KDR mRNA expression would be helpful for a complete understanding not only of the differences in synovial neovascularization in RA and OA, but also of the pathogenesis of RA.

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≪難治性病態の治療戦略≫
腸管 Behçet 病

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はじめに●

　Behçet 病(Behçet's disease)は，再発性口腔内アフタ性潰瘍，皮膚症状，外陰部潰瘍，眼病変(ぶどう膜炎)を四大主症状とする原因不明の炎症に基づく症候群である．本症は，四大主症状をすべて示す完全型とそうでない不全型とに分類される．一方，特殊病型として，消化管の潰瘍性病変を示す腸管 Behçet 病，大小の動静脈の病変をきたす血管 Behçet 病，脳幹・小脳・大脳白質の病変を主体とする神経 Behçet 病，の3型が定義されている．Behçet 病の臓器病変としてはこれらの3つの特殊病型がきわめて重要であり，ときとして生命予後を左右する場合も少なくない．

　本稿においては，これら Behçet 病の特殊病型の中で，腸管 Behçet 病について概説したい．

腸管 Behçet 病の臨床的特徴●

1. 疫学的事項

　Behçet 病発症後, 数年を経たものに多い[1]. 1991年の厚生省特定疾患ベーチェット病調査研究班の疫学調査の結果では，消化管病変の合併率は全Behçet 病患者の 15.5% であった[2]. 発症頻度に男女差はなく，発症のピークは 40 歳前後である[3]. HLA-B 51 の陽性率は 43.5% で，むしろ Behçet 病全体よりも低い傾向を示している[2].

2. 臨床症状と病理組織所見

　Behçet 病においては食道から直腸までのすべての部位に潰瘍性病変を生じうる[3]. 食道潰瘍は，Behçet 病の発症早期よりみられ，嚥下痛・嚥下困

Fig. 1.　Behçet 病患者にみられた食道潰瘍

難をきたす(Fig. 1). また，まれにその治癒後に食道の狭窄を残すことがある．一方，下部消化管の病変(いわゆる腸管 Behçet 病)は，Behçet 病発症後数年を経て出現する遅発性病変であり，定型的には回盲部に深い潰瘍を形成する(Fig. 2). 腹痛・下血・腹部腫瘤を示し，発熱を伴うこともある．穿孔を起こす場合もあるので注意が必要である．このような定型的回盲部病変のほかに，潰瘍性大腸炎様の病変などを示す場合もみられる(Fig. 3). 近年はこうした非定型病変の割合が増加する傾向がみられる[3]. Fig. 4 に，当教室で 1989～1999年のあいだに経験した 26 症例の病変部位の分布を示す．

　病理組織学的には非特異的な炎症像が認められる．定型的な症例では，潰瘍部粘膜下層の小静脈に内膜増殖や血栓形成を認め, microangiography

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