INTRODUCTION

Bone marrow endothelial cells (BMECs) support the proliferation and differentiation of hematopoietic progenitors via production of various cytokines and also possibly via physical contact (1). In addition, their anatomic location suggests that BMECs play an important role in hematopoietic cell trafficking (2, 3). Therefore, BMECs should somehow differ from endothelial cells outside the bone marrow, and be specialized for their expected role. Indeed, BMECs support the adhesion and migration of hematopoietic progenitors better than human umbilical vein endothelial cells (HUVECs) or lung-derived endothelial cells (4-6). The sulfation patterns of heparan sulfate derived from human BMECs differ from those of HUVECs (7). The expression of adhesion molecules of primary human BMECs and their regulation by cytokines do not differ much from those of HUVECs (4).

Stromal cell-derived factor-1 (SDF-1), a CXC chemokine constitutively expressed and produced by bone marrow stromal cells, induces chemotaxis of both committed and primitive hematopoietic progenitors via its receptor, CXCR4 (8-11). A concentration gradient of SDF-1 across the endothelium in the bone marrow seems to be the major mechanism for bone marrow homing of hematopoietic stem cells and progenitor cells (12, 13). It has been reported that murine BMECs express SDF-1 mRNA and that its conditioned medium has chemotactic activity, which is abrogated with anti-SDF-1 antibody, suggesting the release of SDF-1 from BMECs (14). Another group, however, failed to find chemotactic activity on hematopoietic progenitors in murine BMEC-conditioned medium, although the cells strongly expressed SDF-1 mRNA and the presence of SDF-1 in human BMECs was demonstrated using immunohistochemistry of bone marrow sections (15, 16). Therefore, it is still not clear whether human BMECs actively release SDF-1 and what role BMEC-derived SDF-1 may play in hematopoietic cell trafficking. It is well known that endothelial cells, including HUVECs, generally express CXCR4 and respond to SDF-1, resulting in cell chemotaxis (17). Signalling via CXCR4 seems to be crucial in both pre- and postnatal angiogenesis (18, 19). However, it is not known whether BMECs also express CXCR4. This study investigated the production and secretion of SDF-1 and the expression of CXCR4 in human BMECs.

MATERIALS AND METHODS

Cells and Cell Lines

BMEC-1 cells were generated by introducing the SV-40 large T antigen into an early passage of primary BMECs (20). Cells were cultured in Medium-199 (Gibco BRL Life Technologies, Grand Island, NY, U.S.A.) with 10 to 20% fetal bovine serum (FBS; Gibco) and passaged weekly. The cells are contact-inhibited and express adhesion molecules like primary BMECs (21). HUVECs were prepared as described previously (21) and cultured in Medium-199 (Gibco) supplemented with 20% FBS, 2 mmol/L L-glutamine, 5 U/mL heparin, 100 IE/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL endothelial cell growth supplements (ECGS; Biomed-
cultural Technologies, Inc. Stoughton, MA, U.S.A.), hereafter referred to as EC medium. Early passages were used in the experiments. Primary human BMECs were isolated using the method of Rafii et al. (4) with some modifications. Bone marrow aspirates were obtained after informed consent from normal donors undergoing bone marrow harvest. Bone marrow spicules, obtained by filtration through a 40-micron filter, were washed and digested with 0.1% collagenase (Sigma, St. Louis, MO, U.S.A.) for 15 min at 37°C. After washing, both loose cells and particles were plated onto gelatin-coated tissue culture flasks (Falcon, Franklin Lakes, NJ, U.S.A.) and grown in EC medium. After reaching confluence, they were passaged and endothelial cells were selected using the MACS system (Miltenyi Biotec, Auburn, CA, U.S.A.). First, the cells were incubated with phycoerythrin (PE)-conjugated anti-CD34 (PECAM-1) monoclonal antibody (MoAb) (BD PharMingen, San Diego, CA, U.S.A.), and then reacted with anti-PE MoAb-coated microbeads (Miltenyi Biotec). PE-positive cells were selected using MACS columns according to the manufacturer’s instructions. Only cultures containing at least 80% von Willebrand factor (vWF)-expressing cells were used in the experiments.

Murine bone marrow stromal cells MS-5 (22) were grown in MEM-α (Gibco) supplemented with 10% FBS and passaged weekly. The cells support the proliferation of primitive human hematopoietic cells in long-term culture (23) and secrete SDF-1 (8). Primary human bone marrow stromal cells were obtained as described previously (24). CD34-negative cells, obtained after purification of CD34+ cells from bone marrow mononuclear cells, were cultured in MEM-α supplemented with 12.5% FBS, 12.5% horse serum (Gibco), 5 × 10⁻³ M 2-mercaptoethanol (Sigma), streptomycin, and penicillin, hereafter referred to as LTC medium. After reaching confluence, adherent cells were harvested and maintained in LTC medium with weekly passage. After three passages, neither CD45+ cells nor PECAM-1+ cells were detected by flow cytometric analysis.

Growth factor-dependent human myeloid leukemia cells, MO7e, were cultured in IMDM (Gibco BRL Life Technologies) supplemented with 10% FBS and 10 ng/mL granulocyte, macrophage colony-stimulating factor (GM-CSF; Lucky Biotec, Daejon, Korea). The cells strongly expressed CXCR4 and responded to SDF-1, resulting in chemotaxis (9).

**Purification of CD34+ Cells**

CD34+ cells were purified from bone marrow obtained from normal donors undergoing bone marrow harvest with informed consent. After Ficol isolation, the mononuclear cells were incubated with anti-CD34 MoAb-coated microbeads (Miltenyi Biotec). Bead-positive cells were isolated using the MACS system according to the manufacturer’s instructions. Only cells with purity of 95% or more in flow cytometric analysis were used in the experiments.

**Flow Cytometry**

A total of 5 × 10⁴ to 1 × 10⁵ cells was incubated at 4°C for 30 min with fluorescein isothiocyanate (FITC)- or PE-conjugated MoAb, and analysed using a Coulter Elite flow cytometer (Coulter Electronics Ltd., Hialeah, FL, U.S.A.). Isotype-identical antibodies served as controls. The monoclonal antibodies used in the study were as follows: FITC-conjugated anti-CD34 MoAb (HPAC-2; Becton-Dickinson, San Jose, CA, U.S.A.), PE-conjugated anti-CXCR4 MoAb (12G5; BD PharMingen), PE-conjugated anti-CD45 MoAb (BD PharMingen), and PE-conjugated anti-CD106 MoAb (VCAM-1; BD PharMingen).

**Reverse Transcriptase-polymerase Chain Reaction (RT-PCR)**

Using Trizol™ (Gibco-BRL, Burlington, Ontario, Canada), RNA was prepared from HUVECs, BMEC-1 cells, MO7e cells, and primary bone marrow stromal cells according to the manufacturer’s instructions. After purification, 1 μg of RNA was reverse-transcribed using SuperScript reverse transcriptase (Gibco-BRL) and universal primer oligo dT15 (Promega, Madison, WI, U.S.A.). One μL of cDNA was added to 24 μL of PCR buffer (Gibco-BRL) supplemented with 2 mmol/L MgCl₂, 0.2 μmol/L specific primers, and 1 U/μL reaction tube of Koma Taq polymerase (Koma International, Seoul, Korea). PCR was performed at 94°C for 1 min, at 55-65°C for 45 sec, and at 72°C for 1 min for 30 cycles on a GeneAmp PCR system (Perkin Elmer, Norwalk, CT, U.S.A.). The following primers were used: CXCR4 sense-AAT CTT CCT GCC CAC CAT CIA CTC C; anti-sense-GGC GTC ACA GAT ATA TCT GTC ATC TGC C; SDF-1 sense-TGA AGG TCG GAG TCA ACG GAT TTG TCT TA; anti-sense-CIT AGG GGA TTT GGA AGT TT; GAPDH sense-TGA AGG TCG GAG TCA ACG GAT TTG GTC; anti-sense-CAT GTG GGC CAT GAG GAC GTC CAC CAC.

**Preparation of Conditioned Medium (CM)**

BMECs, HUVECs, MS-5, and primary human bone marrow stromal cells were grown in the proper medium for each in T25 culture flasks. After reaching confluence, the cultures were thoroughly washed, and 3 mL of serum-free medium X-VIVO (Biowhittacker, Walkersville, MA, U.S.A.) was added to each flask. After a 72-hr incubation at 37°C, the supernatants were harvested, filtered using 2 μm membranes, and stored at 4°C until used. As indicated, cytokines were added at the beginning of the cultures for the preparation of conditioned medium. The following cytokines were used: interleukin-1β (IL-1β; 10 ng/mL), interleukin-3 (IL-3; 50 ng/mL), interleukin-6 (IL-6; 20 ng/mL), stem cell factor (SCF; 100 ng/mL), interferon-gamma (IFN-γ; 100 U/mL), tumour necrosis factor-alpha (TNF-α 10 ng/mL), transforming growth factor-beta 1 (TGF-β
1; 10 ng/mL) (all of the above were purchased from R&D Systems), granulocyte colony-stimulating factor (G-CSF; 100 ng/mL) (a gift from Kirin, Tokyo, Japan), and GM-CSF (100 ng/mL). As indicated, heparinase (Sigma) was added at concentrations of 10, 20, 50, or 100 U/mL.

Transmigration Assay

The Transwell™ system (Corning-Costar, Cambridge, MA, U.S.A.) was used, as previously described (11). CD34+ cells or MO7e cells (2-2.5 × 10^5 cells per well) were loaded into the upper chamber of a 24-well Transwell with a 3 μm microporous membrane, and SDF-1, cytokines, or conditioned media were added to the lower chamber. The cells were allowed to migrate into the lower chamber for the indicated period. For blocking experiments, CD34+ cells were pre-incubated with 200 μg/mL of PTX (Sigma) at 37°C for 2 hr.

Enzyme-linked Immunosorbent Assay (ELISA)

The concentrations of SDF-1 and SCF in conditioned media were measured using commercial ELISA kits (R&D Systems) according to the manufacturer’s instructions. Optical density was measured using a spectrophotometer (Molecular Device Co., Sunnyvale, CA, U.S.A.). The standard curves and levels of SDF-1 or SCF were obtained using the program Softmax™ (Molecular Device Co.).

Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded human bone marrow sections.

Anti-human CXCR4 monoclonal antibody (R&D Systems) was diluted (1:200), and immunostaining was performed using an LSAB kit (DAKO, Carpinteria, CA, U.S.A.). After pre-incubation with goat serum for 15 min, bone marrow sections were incubated overnight with anti-CXCR4 antibody at 4°C in a humid chamber. After washing, the slides were incubated for 30 min with a linking solution containing biotinylated goat anti-mouse IgG, and sequentially incubated with streptavidin peroxidase for 30 min and with 3,3-diaminobenzidine (DAB) for 5 min. Slides were counterstained with Mayer’s hematoxylin. Isotype-matched antibody was used as a negative control.

Statistical Analysis

The results are expressed as the means ± SD of three or more independent experiments. Data were analysed using Student’s t-test for paired or unpaired samples. A p value of less than 0.05 was considered significant.

RESULTS

Expression of CXCR4 and SDF-1 in BMECs and HUVECs

In flow cytometric analyses, BMEC-1 cells did not express CXCR4. In contrast, HUVECs strongly expressed CXCR4, and this was highly up-regulated by IL-1β as shown in previous studies (Fig. 1) (17). RT-PCR also revealed that BMEC-1 cells did not express CXCR4 mRNA, whereas HUVECs strongly expressed it. Incubation of BMEC-1 cells with var-
ious cytokines for up to 40 hr did not induce the expression of mRNA or cell surface protein for CXCR4. Interestingly, CXCR4 was consistently detected in the endothelial cells lining the sinusoids, venules, and arterioles of the bone marrow using immunohistochemistry (Fig. 2). SDF-1 mRNA was detected in BMEC-1 cells by RT-PCR, but not in HUVECs. Its expression, however, was much less than that of primary human bone marrow stromal cells (Fig. 3).

**Chemoattractive Activities of BMEC CM and HUVEC CM on Hematopoietic Progenitor Cells**

While 0.3 ± 0.2% of bone marrow CD34+ cells migrated in controls in 4 hr, BMEC-1 CM induced the migration of 2.5 ± 1.5% of the cells; the migration, however, did not differ from the value for HUVEC CM (1.7 ± 0.5%, p=0.1), but was far less than for MS-5 CM (19.6 ± 11.0%, p<0.01). Pretreatment of CD34+ cells with pertussis toxin did not affect BMCE-1 CM- or HUVEC CM-mediated migration, but it markedly inhibited the migration of cells induced by MS-5 CM (Fig. 4). To clarify whether cytokines modulate the chemoattractive activities of BMEC-1 cells, the cells were incubated with some cytokines and the chemoattractive activity of the CM was measured. IL-1β-treated CM had increased and TNF-α-treated CM had markedly decreased chemoat-
Production of SDF-1 in Bone Marrow Endothelial Cells

The data are presented as the mean ± SD of the percentages of transmigration from three independent experiments. *, p<0.05 compared to control.

Measuring SDF-1α and SCF in BMEC CM and HUVEC CM

The concentrations of SDF-1α in three-day-old CM of endothelial cells and bone marrow stromal cells were measured by ELISA. SDF-1α was not detected in HUVEC CM, even after various cytokine treatments. BMEC-1 CM and primary human BMEC CM contained 1,710.7 ± 204 and 1,050 ± 153 pg/mL SDF-1α, which were much lower than with primary bone marrow stromal cell CM (29,536 ± 532 pg/mL, p<0.001) or MS-5 CM (101,791 ± 9,045 pg/mL, p<0.001) (Table 1). It was markedly increased by treating BMEC-1 cells with IL-1β and SCF, and abrogated by treatment with TNF-α. BMEC-1 CM and HUVEC CM contained 324 ± 24 and 269 ± 14 pg/mL SCF, respectively; both of which were increased by treating the cells with IL-1β or TNF-α (Table 2). Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes (25), and SDF-1 binds to glycosaminoglycan on the cell surface (26). To clarify whether SDF-1 is present on BMEC-1 cells, heparinase, which digests heparan glycan on the cell surface (26), was added to BMEC-1 cell culture, and the SDF-1 concentration in the culture supernatant was measured. The addition of heparinase at the concentration of up to 100 U/mL increased the concentration of SDF-1α in the culture supernatant in a dose-dependent manner by up to 32.5 ± 5% (data not shown), indicating that SDF-1 was present on the cell surface and released from glycosaminoglycan by enzymatic cleavage.

DISCUSSION

SDF-1 and CXCR4 interaction is involved in both normal cardiovascular development (18) and postnatal angiogenesis.

Table 1. SDF-1α and SCF levels in conditioned medium measured using ELISA

| Conditioned media | SDF-1α (pg/mL) | SCF (pg/mL) |
|-------------------|----------------|-------------|
| BMEC-1            | 1,710±204      | 324±24      |
| Primary BMEC      | 1,050±153      | 478±57      |
| HUVEC             | BDL            | 269±68      |
| Primary BMSC      | 29,536±532     | 1,665±92    |
| MS-5              | 101,791±9,045  | NT          |

Data are representatives of three independent experiments (mean ± SD). BMEC, bone marrow endothelial cells; BMSC, bone marrow stromal cells; BDL, below detectable levels; NT, not tested.

Table 2. SDF-1α and SCF levels in cytokine-treated endothelial cell conditioned medium measured using ELISA

| Cytokines added | SDF-1α (pg/mL) | SCF (pg/mL) |
|-----------------|----------------|-------------|
|                  | BMEC-1         | HUVEC       | BMEC-1         | HUVEC       |
| No              | 1,710±204      | BDL         | 324±24         | 269±14      |
| IL-1β (10 ng/mL)| 6,151±41*      | 747±27*     | 605±68*        |             |
| IL-3 (50 ng/mL) | 1,537±122      | 307±17      | NT             |             |
| IL-6 (20 ng/mL) | 1,984±204      | BDL         | NT             | NT          |
| IL-6 (20 ng/mL) | 2,157±205      | BDL         | 360±38         | 341±14      |
| GM-CSF (100 ng/mL)| 1,058±205    | BDL         | NT             | NT          |
| SCF (100 ng/mL) | 4,068±368*     | BDL         | NT             | NT          |
| IFN-γ (100 U/mL)| 1,276±82       | 324±35      | 302±21         |             |
| TNF-α (10 ng/mL)| 278±81*        | BDL         | 757±25*        | 583±43*     |
| TGF-β (10 ng/mL)| 1,492±245      | BDL         | 305±24         | NT          |

Data are representatives of three independent experiments (mean ± SD). *p<0.05 compared to no-cytokine-treatment groups. BDL, below detectable level; NT, not tested.
(19). In this study, we showed that endothelial cells lining the bone marrow vasculature also express CXCR4, although it is not detected in cultured BMEG or BMEC-1 cells. It is not clear why cultured BMECs lose their ability to express CXCR4, whereas HUVECs preserve it well. Recently, time-dependent changes in CXCR4 expression and localization during extracellular matrix-dependent endothelial tube formation of HUVECs were observed (27). BMECs may lose their ability to express CXCR4 when they are not in the vasculature.

Skin endothelial cells produce and secrete SDF-1 (28). Peled et al. (15) found no evidence that HUVECs produced SDF-1, while Salvucci et al. (27) observed SDF-1 production by HUVECs, especially in the presence of vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF). We could not detect SDF-1 mRNA in HUVECs. Furthermore, SDF-1 was not detected in culture supernatant of HUVECs incubated in serum-free conditions using ELISA. Taken together, SDF-1 production by HUVECs is minimal and is dependent on the presence of certain cytokines, such as VEGF or bFGF.

Imai et al. (14) reported that murine BMECs expressed SDF-1 mRNA, and that the conditioned medium had chemotactic activity that was blocked by anti-SDF-1 antibody. However, they did not compare the chemoattractiveness of BMEC CM and bone marrow stromal cell CM. Peled et al. (15) proved the presence of SDF-1 in BMECs in human bone marrow sections using immunohistochemistry. However, they could not find chemoattractive activity in murine BMEC CM, although the cells expressed SDF-1 mRNA. Therefore, it remains unclear whether human BMECs secrete SDF-1, and whether the amount of SDF-1 released from BMECs is comparable to that from bone marrow stromal cells. In this study, we showed that BMECs secrete soluble chemoattractive factors for hematopoietic progenitors. BMEC-1 CM in the lower chamber of the Transwell™ induced migration of CD34+ cells. The failure of pretreating the cells with pertussis toxin to block transmigration indicates that SDF-1 is not a predominant chemoattractive factor released from BMECs. In agreement with this, much less SDF-1 was secreted from BMECs than from primary human bone marrow stromal cells or a murine bone marrow stromal cell line, MS-5. The expression of SDF-1 mRNA by BMEC-1 was also much lower than by bone marrow stromal cells. These results indicate that BMECs are not the major source of the high concentration of SDF-1 in the bone marrow microenvironment, and strengthens the possibility that SDF-1 produced by BMECs has additional roles. It has been shown that SDF-1 and SCF cooperate in inducing chemotaxis and chemokinesis of hematopoietic progenitor cells (9), possibly by sharing common signalling pathways (29). The chemoattractive activity produced by BMECs might be due to the additive or synergistic actions of some chemoattractive factors. We showed that the production and secretion of SDF-1 from BMECs is regulated by cytokines, including some hematopoietic growth factors. Further studies on the implications of modulation of SDF-1 production by BMECs are needed. IL-1α-treated BMEC-1 CM had enhanced chemoattractive activity and TNF-α-treated BMEC-1 CM had attenuated chemoattractive activity on MO7e cells. It remains unclear whether the modulation of SDF-1 production or secretion by the cytokines could be responsible for the changes in chemotraction.

It has been suggested that transendothelial migration of hematopoietic progenitors is characteristic of BMECs, based on the finding that murine BMECs supported the transmigration of hematopoietic progenitors, while lung endothelial cells did not (6). This is untrue, since HUVECs support adhesion and both spontaneous and SDF-1-induced transendothelial migration of hematopoietic progenitors to some extent (16). It has been suggested that SDF-1, especially the surface-bound form, activates the adhesion molecules of hematopoietic progenitors, thereby enhancing adhesion of the cells to endothelial cells (15, 16), although a contradictory report found that SDF-1 suppresses the cytokine-induced adhesion of hematopoietic progenitors to immobilized fibronectin (30). We showed that incubating BMEC-1 cells with heparinase increased the SDF-1 concentration in the culture supernatant, indicating the presence of SDF-1 on the cell surface. Indeed, SDF-1 antibody decreased the adhesion of hematopoietic progenitors to BMECs (14), and pretreatment of HUVECs with SDF-1 enhanced the adhesion of hematopoietic progenitor cells (16). Taken together, it is possible that BMEC-derived SDF-1 enhances the transendothelial migration of hematopoietic progenitor cells.

In conclusion, human BMECs express CXCR4 in vivo and produce and release small amounts of SDF-1, which might favor bone marrow endothelium, with better transmigration of haematopoietic cells.

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