Human Endothelial Cells Regulate Survival and Proliferation of Human Mast Cells

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Abstract

Mast cells (MCs) are immunoregulatory and inflammatory tissue cells preferentially located around blood vessels. Since endothelial cells have been suggested to regulate MC functions, we analyzed MC–endothelial cell interactions in vitro by performing coculture experiments with purified human intestinal MCs and human umbilical vein endothelial cells (HUVECs). We found that HUVECs provide signals allowing MCs to survive for at least 3 wk and to proliferate without addition of cytokines; otherwise all MCs died. HUVEC-dependent MC proliferation was more pronounced than that induced by stem cell factor (SCF), known to act as an MC growth factor both in vitro and in vivo. After coculture with HUVECs, most MCs were of the tryptase and chymase double-positive phenotype (MC_{TC}). Transwell experiments suggested that the HUVECs' effects on MCs are not mediated by soluble factors. HUVEC-dependent MC adhesion and proliferation were inhibited by neutralizing antibodies directed against SCF and vascular cell adhesion molecule (VCAM)-1 expressed on HUVECs, and c-kit and very late antigen 4 (VLA-4) on MCs. The data suggest that two mechanisms (membrane-bound SCF/c-kit and VCAM-1/VLA-4) are involved in human MC–endothelial cell interactions. In conclusion, our study provides evidence that endothelial cells regulate MC survival and preferentially support human MC_{TC} development.

Key words: adhesion • fibroblast • integrins • intestinal mucosa • stem cell factor

Introduction

Human mast cells (MCs)[1] are found predominantly in tissues forming an interface between the host and external environment, such as skin, respiratory, and gastrointestinal mucosa. MCs are also located in muscular organs such as the heart and uterus, and around blood vessels in general, which form a "barrier" between host tissue and blood stream. Most MCs of the human body are located in gut mucosa, which contains in their lamina propria ~2–3% MCs. The density of MCs may be substantially increased at sites of inflammation, wound healing, or tissue fibrosis (1–4). MCs are recruited from poorly characterized bone marrow–derived progenitors circulating in blood and entering the tissue where final maturation is achieved (5). We found that after maturation, MCs may retain the capacity to proliferate after cytokine stimulation, suggesting that MC density in tissue is dependent on local MC proliferation and MC progenitor influx, which both could be regulated by endothelial cells and other cell types (6).

In previous studies we presented methods for the isolation and purification of human intestinal MCs and found that these cells maintain in culture if stem cell factor (SCF) has been added to the culture medium (7, 8). In the absence of SCF, all MCs die within a few days, indicating that SCF is a crucial and unique survival factor for human intestinal MCs. SCF exists in a soluble and a membrane-bound form generated by alternative splicing, and is produced by different cell types, including endothelial cells expressing both variants of SCF as well as c-kit, the SCF receptor (9–13). We found that upon stimulation of endothelial cells with the proinflammatory cytokines IL-1α and TNF-α, SCF ex-

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[1]*Abbreviations used in this paper:* BrdU, 5-bromo-2′-deoxyuridine; FB, fibroblast; HUVEC, human umbilical vein endothelial cell; ICAM, intracellular adhesion molecule; MC, mast cell; MC_T, tryptase-positive MC(s); MC_{TC}, tryptase and chymase double-positive MC(s); mSCF, membrane-bound SCF; SCF, stem cell factor; sSCF, soluble SCF; VCAM, vascular adhesion molecule; VLA-4, very late antigen 4.

pression is upregulated, whereas that of c-kit is downregulated (14). These data suggest that other cell types, e.g. MCs, may be attracted by endothelial-derived SCF. Similar findings were obtained for IL-1α and IL-6, the expression of which is induced in endothelial cells triggered by LPS and further enhanced by IL-1β and TNF-α (15). Interestingly, MCs could be involved in the regulation of SCF expression in endothelial cells, since we and others reported that human MCs produce multiple cytokines, including IL-1β and TNF-α, particularly if the cells were stimulated with bacterial products such as LPS or by IgE receptor cross-linking (16). Furthermore, it was shown that MC granules containing IL-1β, TNF-α, and other factors regulate IL-6 production in endothelial cells (17). These in vitro findings together with the morphological observation that MCs are typically located around blood vessels suggest a bidirectional interaction between MCs and endothelial cells.

To further elucidate the mechanism of interaction between MCs and endothelial cells, we established a coculture system using highly purified human intestinal MCs and human umbilical vein endothelial cells (HUVECs). We found that MCs keep alive for many weeks in the absence of any exogenous growth factor if cultured on an endothelial cell layer. Endothelial cell–dependent MC survival is mediated by SCF and adhesion molecules such as vascular cell adhesion molecule (VCAM)-1. The data suggest that endothelial cells play an important role in regulating not only MC influx but also MC development and function in tissue.

Materials and Methods

Reagents and Buffers. The following cytokines were used for cell culture and/or stimulation: SCF (25 ng/ml; provided by Dr. L. Souza, Amgen, Thousand Oaks, CA), IL-1β (10 ng/ml; PeproTech), IL-4 (10 ng/ml; PeproTech), and TNF-α (10 ng/ml; PeproTech). The concentrations indicated above were found to be maximally effective in our assays as determined in previous experiments (6–8). MC culture medium is RPMI 1640 containing 10% heat-inactivated FCS (Biochrom), 25 mM Hepes (Sigma-Aldrich), 2 mM L-glutamine, 100 μg/ml streptomycin, 100 μg/ml gentamycin, 100 U/ml penicillin, and 500 ng/ml fungizone (all from Life Technologies).

Human MC Isolation and Purification. Human intestinal MCs were isolated from a surgical specimen (macroscopically normal tissue) using a four-step enzymatic method as described previously (7, 8). MCs were partially purified up to 90% via selection of c-kit–positive cells by magnetic cell separation (MACS™ system; Miltenyi Biotec). The partially purified MCs were used for MC–HUVEC or MC–fibroblast (FB) coculture or for MC monoculture. These MC monocultures were supplemented with growth factors such as IL-4 and/or SCF, or with HUVEC supernatants/sonicates. Further purification of the MCs to 98–100% was achieved by a 2-wk cell culture in MC culture medium supplemented with 25 ng/ml SCF and 10 ng/ml IL-4 as described (6, 8). The highly purified MC preparations were washed after 14 d of culture, resuspended in new MC culture medium, and used for stimulation with HUVEC supernatants or sonicates and for the MC–HUVEC adhesion assay (see below).

Human FB Isolation and Purification. Human intestinal FBs were isolated from tissue dispersions obtained after enzymatic digestion as described for human intestinal MCs (7). The cell suspension containing 0.5–2% FBs was seeded into culture flasks and cultured in MC culture medium. Contaminating nonadherent cells were removed by changing the medium every 48–72 h. The adherent FBs were sub-confluent after 5 d and were harvested after a 5-min incubation of the cells at 37°C with a buffer containing 0.25% trypsin and 0.02% EDTA (Biochrom). The FBs were washed and seeded into new 80-cm^2 flasks. The FB preparations did not express CD31 surface marker (18) or von Willebrand factor (14; FACS® analysis, data not shown), and thus were not contaminated by endothelial cells.

HUVEC Isolation and Culture. HUVECs were isolated as described in detail by Jaffe and coworkers (19). Freshly isolated cells were cultured in HUVEC culture medium consisting of MC culture medium and M199 medium (Life Technologies) in a 1:2 ratio. The culture flasks were coated with 0.1% gelatine (Sigma-Aldrich). After 24 h, nonadherent cells were removed by changing the culture medium and new medium was added to the adherent cells. HUVECs were grown to confluence and were passaged two to four times. Endothelial cell type was confirmed by FACS® analysis of von Willebrand factor and CD31, which were both expressed by almost all cells (data not shown). HUVECs were used for coculture with MCs or for the preparation of HUVEC supernatants, HUVEC sonicates, and HUVEC RNA before and after stimulation of the cells with 10 ng/ml IL-1β or TNF-α for 4 or 24 h.

MC–HUVEC Coculture Assay. HUVECs obtained after the second to fourth passage were cultured in MC culture medium in 24-well plates coated with 0.1% gelatine for at least 4 h to allow adhesion on the surface of the plates. MCs purified by MACS™ were seeded onto subconfluent HUVEC monolayers with or without separation of the two cell types using Transwell membranes (0.4-μm pore size; Nunc). For some conditions MCs were cocultured with HUVECs in the presence or absence of cytokines (as described above) or neutralizing mAbs (anti–human SCF, anti–human IL-3, and anti–human IL-3 receptor α chain [R&D Systems]; anti-CD29, clone Lia1/2, anti-CD49d, clone HP2/1, and anti-CD106, clone 1G11, all used at 5 μg/ml [Coulter]). Total coculture time was up to 21 d. Every week, half of the medium was changed and adherent and nonadherent MCs and HUVECs were harvested using a cell scraper. All cells were put on new HUVEC monolayers to avoid confluence and subsequent cell death of the HUVECs. MC numbers were determined by cell counting. The MC countings were confirmed by determination of total cellular histamine content measured after cell lysis. The MC–HUVEC cocultures were also used for adhesion assay after a coculture time of 15 min to 6 h (see below).

MC–FB Coculture Assay. FBs in the second to fourth passage were cultured in MC culture medium for at least 4 h to allow adherence. Subconfluent FBs were cocultured with MCs as described above for MC–HUVEC cocultures.

RNA Isolation and Reverse Transcriptase PCR. Total RNA was isolated from 2×10^5 human intestinal MCs (98–100% purity) and from 2×10^5 HUVECs (100% purity) using the RNeasy Mini Kit (QIAGEN). Genomic DNA was removed by treating 200 ng of total RNA for 15 min at 37°C with 10 U/ml RNase-free DNase (Promega). After denaturation for 10 min at 70°C, cDNA was transcribed for 1 h at 37°C by adding Superscript™ reverse transcriptase (Life Technologies) and 20 pmol oligo dT primers (Amersham Pharmacia Biotech). For each PCR 1/10 vol of cDNA was used. PCR was performed in 35 cycles (60 s at 94°C, 80 s at 60°C, and 70 s at 72°C) using 2.5 U/ml Taq DNA polymerase (Life Technologies). Intron-spanning sets of primers

were used to amplify cDNA specific for: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sense: 5′-ACCACAGTCCATGCCATCAC-3′; 20 mer and antisense: 5′-TCCACCACCCTGTTGCTGTA-3′; 20 mer, fragment size 452 bp), IL-4 (sense: 5′-CGGACACAAGTGCGATATCACC-3′, 22 mer and antisense: 5′-CCAACGTACTCTGGTTGGCTTCC-3′; 23 mer, fragment size 331 bp), soluble SCF (sSCF) (sense: 5′-GGGCTGGATCGCAGCGC-3′, 17 mer and antisense: 5′-CTCCACAAGGTCATCCAC-3′; 18 mer, fragment size 276 bp), and membrane-bound SCF (mSCF) (sense: 5′-CTTCAACATTAAGTCCCTGAG-3′, 21 mer and antisense: 5′-GTCTAGGCTGGAGTCTCC-3′; 18 mer, fragment size for mSCF 359 bp and for sSCF 275 bp). PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

MC Flow Cytometry. MCs (98–100% purity) were washed twice in PBS buffer containing 0.1% BSA and 0.1% NaN_3. The pellet was resuspended in the same buffer supplemented with 250 μg/ml rabbit IgG (Dianova) for blocking Fc receptors. For each condition, 1×10^5 MCs were labeled with primary mAbs directed against CD11a (clone 25.3.1; Coulter), CD11b (clone BEAR1; Coulter), CD11c (clone B-ly6; Becton Dickinson), CD15s (clone 2H5; Becton Dickinson), CD18 (clone MHM23; Dako), CD29, CD31 (clone HC1/6; Southern Biotechnology Associates, Inc.), CD33 (clone HIM3-4; Becton Dickinson), CD43 (clone DFT1; Dianova), CD49a (clone HP2B6; Coulter), CD49b (clone AK7; Becton Dickinson), CD49c (clone P1B5; Becton Dickinson), CD49d, CD49e (clone SAM1; Coulter), CD49f (clone GoH3; Becton Dickinson), CD50 (clone TU41; Becton Dickinson), CD51 (clone 23C6; Southern Biotechnology Associates, Inc.), CD54 (clone 84H10; Coulter), CD61 (clone VI-PL2; Becton Dickinson), CD62E (clone 1.2B6; Southern Biotechnology Associates, Inc.), CD62L (clone FMC46; Southern Biotechnology Associates, Inc.), CD62P (clone AK-4; Becton Dickinson), CD63 (clone H5C6, Becton Dickinson), CD102 (clone BMS109; Bender Systems), CD106, CD107a (clone H4A3; Becton Dickinson), CD117 (clone YB5.B8; Becton Dickinson), CD162 (clone KPL-1; Becton Dickinson), β7 (clone FIB504; Becton Dickinson), and anti–human SCF (reference 20; clone 4B10). Appropriate isotype controls (mouse IgG1, IgG2a, IgG2b, IgM, and rat IgG) were used (all from Southern Biotechnology Associates, Inc.). After an incubation time of 30 min at 4°C, cells were washed twice and stained for 30 min at 4°C with the secondary Abs (FITC- or PE-conjugated goat anti–mouse IgG1, IgG2a, IgG2b, IgM, or goat anti–rat IgG; all from Southern Biotechnology Associates, Inc.). FACS® analysis was performed using the FACSCalibur™ system and CELLQuest™ software (Becton Dickinson).

HUVEC and FB Flow Cytometry. Before staining, adherent HUVECs or FBs were detached from the surface of the culture flasks by treatment at 37°C with a solution containing 20 mmol/liter Hepes, 150 mmol/liter sodium chloride, and 500 nmol/liter EDTA in PBS, pH 7.2, for 20 min at 37°C. Remaining adherent cells were harvested using a cell scraper and washed twice in HUVEC or MC culture medium. For each condition, 2×10^5 HUVECs or FBs were stained with primary mAbs as described above.

Adhesion Assay. Human MCs were cocultured with HUVECs as described for the MC–HUVEC coculture assay. After a variable coculture time (15 min to 6 h), nonadherent cells were washed away and MC numbers of the remaining adherent cells were determined by cell counting and cell differentiation of smears stained with May-Grünwald/Giemsa (Merck). To inhibit MC adhesion to HUVECs, different neutralizing mAbs were added into the MC culture medium, such as anti–human SCF (5 μg/ml; R&D Systems), anti-CD18, -CD29, -CD51, -CD54, -CD106, -CD49d, -CD62P, -CD62L, -CD62E (all at 5 μg/ml; the same Abs were used for flow cytometry), and anti–c-kit (reference 11; clone SR1, 1:100). Appropriate isotype controls (5 μg/ml of mouse IgG1 and IgG2b, and goat IgG; Southern Biotechnology Associates, Inc.) were included.

Scanning Electron Microscopy. HUVECs (second to fourth passage) were cultured on 0.1% gelatine-coated glass cover slides for at least 24–48 h to allow confluence of the cells. Then, MCs were seeded onto the HUVEC monolayer and cocultured for 4 h. The adherent MCs were washed twice with pre-warmed PBS buffer, fixed with Karnovsky's fixative (2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4) for 2 h, and rinsed overnight in 0.1% cacodylate buffer. The cells were dehydrated in graded series of ethanol concentrations (30, 50, 70, and 90% each for 5 min two times and 100% each for 5 min four times) and air-dried at room temperature. To detect the surface structure of the cells, they were coated with gold-palladium before analysis using a scanning electron microscope (model 505; Phillips).

Immunocytochemistry. Immunocytochemistry was performed using mAbs against human tryptase (mouse IgG1, 230 ng/ml; Chemicon) and human chymase (mouse IgG1, 100 ng/ml; Chemicon) as primary Abs (overnight incubation at 4°C) and the streptavidin-biotin detection system (Histostain-Plus kit; Zymed Laboratories) as described (6–8). In all experiments, an appropriate isotype control Ab was included (mouse IgG1, 230 ng/ml; Southern Biotechnology Associates, Inc.). The number of tryptase-positive (MC_T) or tryptase and chymase double-positive MCs (MC_{TC}) was determined by analyzing 600 MCs.

SCF ELISA. SCF was measured in supernatants of HUVEC or FB monolayers (10^6 cells/ml) incubated with or without 10 ng/ml TNF-α for 4 h using Quantikine® human SCF assay (R&D Systems). The detection limit of the assay was 9.0 pg/ml.

Proliferation and Apoptosis Assays. MCs that have been cultured under different conditions (SCF alone, SCF and IL-4, coculture with HUVEC, and coculture with FB) were analyzed for proliferation and apoptosis after 7 and 14 d of culture. MC proliferation was assessed by quantification of cells incorporating 5-bromo-2′-deoxyuridine (BrdU) that was added to the cell culture at 3 μg/ml for 3 d (during days 4–7 and days 11–14) following the manufacturer's instructions (Amersham Pharmacia Biotech). Two negative controls were included (MCs not incubated with BrdU and stained with anti-BrdU Ab, and MCs incubated with BrdU but stained with an isotype control mouse IgG2a Ab) yielding negative results (6). In addition, MC proliferation was analyzed after 7 and 14 d of coculture with HUVECs or FBs by counting cells staining positive after immunocytochemistry using an Ab directed against the human nuclear cell proliferation antigen Ki67 (mAb MIB-1 at 5 μg/ml; Dianova) and an isotype control Ab (mouse IgG1) as described (6). MC apoptosis was analyzed using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling in situ cell death detection kit for immunocytochemistry (Boehringer). MC apoptosis was further examined by assessing MC binding of annexin V after incubation of MCs for 15 min with 50 ng/ml annexin V–FITC using the TACS™ Annexin V-FITC Apoptosis detection kit for FACS® analysis (R&D Systems).

Statistics. Data were expressed as mean values ± SD, if not indicated otherwise. Statistical analysis was performed using the two-tailed paired *t* test. A $P < 0.05$ was considered to be statistically significant.

Results

Survival and Proliferation of MCs Cultured in the Presence of HUVECs. Confirming our recently published results, we found that purified human intestinal MCs maintain in culture for several weeks if the culture medium was supplemented with SCF (Fig. 1). In the absence of SCF, all cells died within 7 d (7). If the culture medium was supplemented with SCF and IL-4, a pronounced proliferation of MCs was observed resulting in an enhanced MC number, whereas IL-4 by itself failed to provide MC survival in culture (6). Most interestingly, Fig. 1 also shows that MCs maintained in culture for up to 3 wk without any cytokine supplementation if MCs were placed on an HUVEC monolayer. These data strongly suggest that HUVECs support MC survival and proliferation, since the number of MCs was higher after 21 d compared with the number at culture start (152 ± 20% MC recovery, $n = 13$). The effect of HUVECs on MC proliferation was higher than that of SCF and more consistent than that of SCF and IL-4. In 4 out of 13 experiments, all MCs died in the presence of SCF, and also in the presence of SCF and IL-4, but not if cocultured with HUVECs. This suggests that the HUVEC-dependent MC proliferation is not only mediated by SCF.

To elucidate the mechanism of HUVEC-dependent MC proliferation, we compared the effects of HUVEC supernatants and sonicates obtained from pure HUVECs cultured for 21 d with that of HUVEC cocultures on MC numbers. All HUVEC supernatants and sonicates failed to provide MC survival if added to MC cultures, even if HUVECs had been stimulated with IL-1β or TNF-α (both at 10 ng/ml, $n = 4$; data not shown). Separation of MCs from HUVECs using Transwell membranes with a pore size of 0.4 μm almost abolished the proliferating effect of HUVECs on MCs (Fig. 2 A). These data strongly suggest that membrane-associated molecules rather than soluble factors mediate the effect of HUVECs on MC survival and proliferation. Fig. 2 B shows that MCs adhere to HUVECs and that adhesion occurs in a time-dependent fashion. Already 15 min after the start of coculture at 37°C, 10 ± 1% of MCs adhered on the cell surface of HUVECs ($n = 3$). After 2 h, 64 ± 12% of MCs adhered to HUVECs as shown in Fig. 3. The binding to HUVECs steadily increased with time up to 98 ± 2% MCs after 6 h. Once adhered to HUVECs, MCs could not be removed from the surface of the HUVECs by repeated washing.

We could confirm by light microscopy and scanning electron microscopy that in MC–HUVEC coculture experiments both cell types are in direct cell–cell contact (Fig. 3, A and B). In many cases, we detected mitotic MCs and HUVECs if both cell types were adjacent (Fig. 3 C), further suggesting that the interaction between the two cell types induces proliferation of MCs and possibly also of HUVECs. This could be confirmed by the incorporation of BrdU in both MCs and HUVECs (Fig. 3, D and E). Furthermore, we studied the MC subtypes that bound to HUVECs and survived in the presence of HUVECs by immunocytochemistry (Fig. 3, F–I). If precultured MCs containing ~20–40% MC_{TC} were cocultured with HUVECs for 4 h, both MC subtypes bound to HUVECs (Fig. 3 F). Analysis of MC subtypes after 14 d of coculture with HUVECs revealed a pronounced change of the MC_{TC}/MC_T ratio of adherent MCs towards 85:15 (Fig. 3, G and H).

Cytokines Involved in MC–Endothelial Cell Interactions. It is unlikely that IL-4 is involved in HUVEC-dependent MC proliferation because we could show by reverse transcriptase PCR that neither purified MCs nor HUVECs expressed IL-4 mRNA under these conditions, and we found by FACS® analysis that both cell types separated after coculture using an EDTA buffer do not contain IL-4 protein

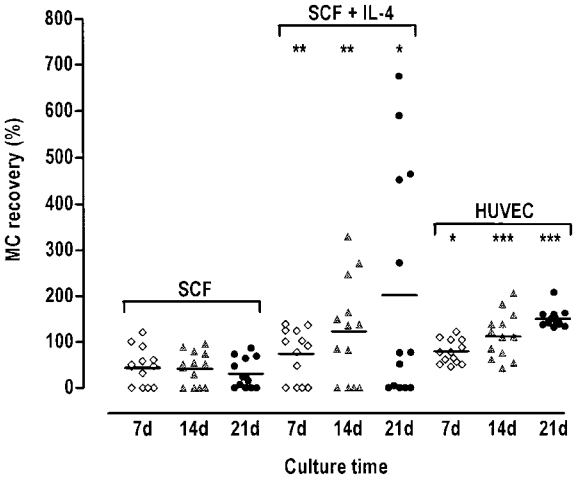

Figure 1. Coculture of human intestinal MCs with HUVECs. MCs were cultured in medium supplemented with SCF (25 ng/ml), or with SCF and IL-4 (10 ng/ml), or on a HUVEC monolayer. After 7, 14, and 21 d, respectively, MC numbers were counted and expressed in percentage of MC numbers at culture start. Each symbol represents one experiment; the bars indicate mean values for each culture condition ($n = 13$). MC recovery after culture with HUVECs or with SCF and IL-4 was compared with that after culture with SCF using the *t* test for paired samples (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

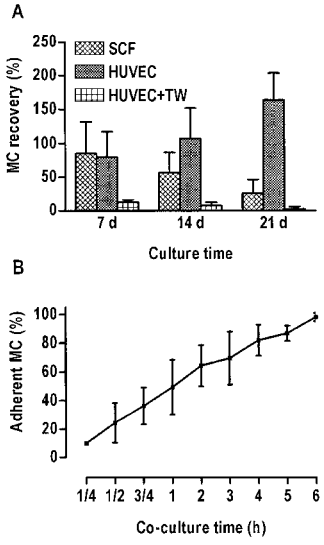

Figure 2. Effect of separation of MCs from HUVECs and time course of MC adhesion to endothelial cells. (A) Effect of separation of MCs from HUVECs using Transwell (TW) plates (0.4-μm pore size) on MC recovery. Means (± SD) of three experiments are shown. (B) Time-dependent increase of percentage of MCs that adhered to HUVECs. MCs were precultured for 2 wk with 25 ng/ml SCF and 10 ng/ml IL-4 before coculture with HUVECs to enhance MC numbers and purity. For details see Materials and Methods. Means (± SD) of three experiments are shown.

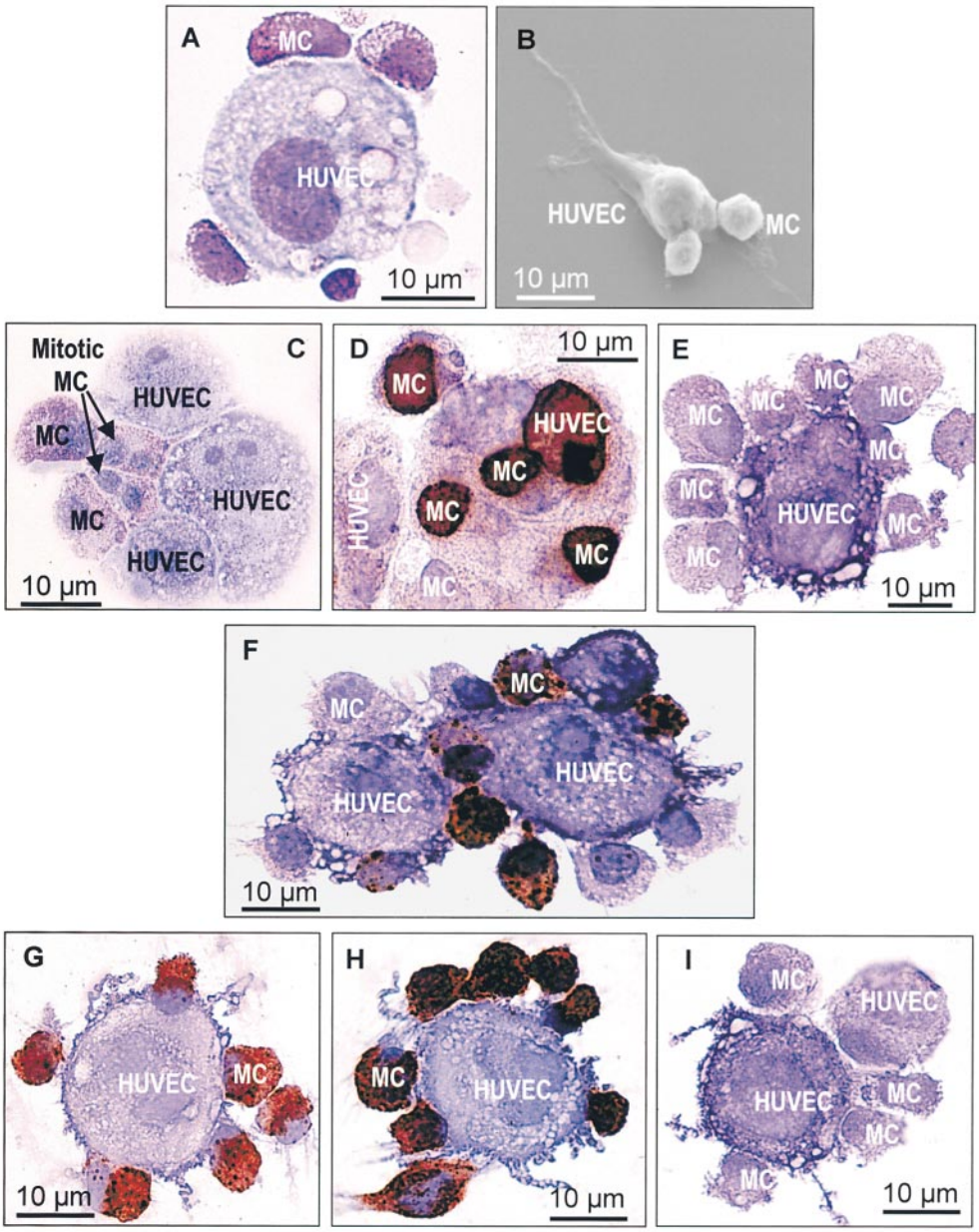

Figure 3. Direct cell–cell contact between MCs and HUVECs. (A) Light microscopy of MCs and HUVECs cocultured for 14 d (May-Grünwald/Giemsa stain). (B) Scanning electron microscopy of MCs and HUVECs cocultured for 4 h showing a close association between both cell types. (C) Light microscopy of mitotic MCs (arrows) and HUVECs cocultured for 14 d (May-Grünwald/Giemsa stain). (D and E) BrdU incorporation shown by immunocytochemistry in MCs and HUVECs cocultured for 14 d. Cells were incubated with 3 μg/ml BrdU for 3 d before harvesting and stained using (D) anti-BrdU Ab or an (E) isotype control mouse IgG2a Ab. (F) Immunocytochemistry using anti-chymase mAb after 4 h of coculture. (G–I) Immunocytochemistry using (G) antichymase mAb, (H) antitryptase mAb, or an (I) isotype control Ab (mouse IgG1, 230 ng/ml) after 14 d of coculture.

(data not shown). Moreover, the competitive IL-4 antagonist RY at a maximally effective concentration of 500 nM failed to affect HUVEC-dependent MC survival (6). IL-3 is also likely not involved in HUVEC-dependent MC proliferation because we found that addition of neutralizing mAbs directed against human IL-3 or IL-3 receptor α chain at maximally effective concentrations to MC–HUVEC cocultures did not affect MC proliferation (data not shown).

On the other hand, SCF is obviously involved in the MC–endothelial cell interaction because HUVEC-dependent MC survival could be totally blocked by adding a neutralizing anti-SCF Ab (Fig. 4 A). However, our observation that HUVECs were generally more effective than SCF in providing MC survival suggests that other signals may be involved. Such signals may enhance MC proliferation in the presence of SCF, but obviously fail to mediate MC survival by themselves. HUVEC-stimulating cytokines like IL-4, IL-1β, or TNF-α (all at 10 ng/ml) added to the coculture system failed to enhance MC proliferation in coculture experiments. In contrast, these cytokines strongly reduced MC numbers (Fig. 4 B) without reducing MC proliferation in MC monocultures supplemented with SCF. Interestingly, neither the addition of SCF nor of SCF and IL-4 to the coculture system significantly enhanced the HUVEC-dependent MC proliferation (data not shown).

Fig. 5 A shows that MC counts are much lower if MCs were cocultured for 21 d with FBs isolated from human intestinal tissue (33 ± 11% MC recovery, $n = 3$) compared with coculture with HUVECs (174 ± 30%). This could be

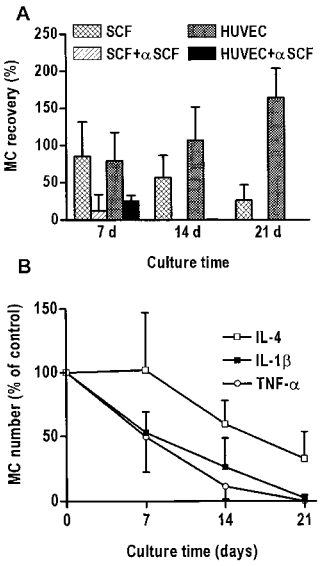

Figure 4. Inhibitory effects of neutralizing anti-SCF Ab and proinflammatory cytokines on the number of MCs cocultured with HUVECs. (A) Effect of neutralizing anti-SCF (αSCF) Ab (5 μg/ml) on the number of MCs cultured in the presence of SCF or HUVECs. Means (± SD) of three experiments are shown. (B) Purified MCs were cocultured with HUVECs in the presence of IL-1β, TNF-α, or IL-4 (all at 10 ng/ml). Data are expressed as percentage of control (MCs cocultured with HUVECs). Data (mean ± SD) of three experiments are shown.

confirmed by studying MC proliferation using the BrdU assay. Fig. 5 B shows that the portion of BrdU-incorporating MCs was much higher in MC–HUVEC cocultures compared with MC–FB cocultures, suggesting that HUVECs, similar to the cytokines, enhance MC numbers by inducing MC proliferation. Moreover, an enhancement of the proliferation marker Ki67 was found in MCs cocultured for 14 d with HUVECs (16 ± 1% Ki67$^+$ MCs after 7 d, 25 ± 12% after 14 d, mean ± SD, $n = 3$) compared with MCs cocultured with FBs (6 ± 2 after 7 d, 6 ± 3% after 14 d). On the other hand, we have no evidence that the increased MC numbers after 14 d of coculture with HUVECs are related to a decrease of MC apoptosis. By using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay in cytospins and the flow cytometric quantification of annexin V–positive cells, we found no significant differences in numbers of apoptotic MCs between the culture conditions (SCF, SCF and IL-4, HUVECs, and FBs; data not shown).

Interestingly in this respect, we found that HUVECs do not release detectable amounts of sSCF into supernatant (<9 pg/10^6 cells). Even after stimulation with TNF-α (10 ng/ml for 4 h), HUVECs produced only small amounts of sSCF (92 ± 80 pg/10^6 cells, mean ± SD, $n = 3$) compared with unstimulated FBs (1,608 ± 156 pg/10^6 cells; Fig. 5 C). These results largely exclude the possibility that intestinal FBs, which were possible contaminating cells in our MC preparation, are significantly involved in MC–endothelial cell interactions. Taken together, our data indicate that mSCF is more effective than sSCF in providing MC proliferation.

We could show by FACS® analysis that HUVECs expressed mSCF on their cell surface. The expression of mSCF on HUVECs slightly increased after 4 h of coculture with MCs (Fig. 6 A). Surprisingly, FBs expressed similar levels of mSCF as HUVECs (Fig. 6 B). In contrast to previous data indicating mSCF expression in skin and cord blood MCs (21), we found no SCF expression in human intestinal MCs (Fig. 6 C), but high levels of c-kit (Fig. 6, D and E). After 4 h of coculture with HUVECs, the intensity

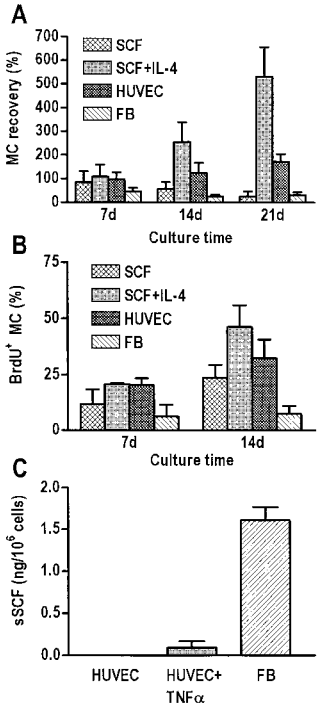

Figure 5. Comparison of the effects of HUVECs and FBs on MC recovery. (A) Purified MCs were cultured for 21 d in medium supplemented with SCF or SCF and IL-4. Alternatively, MCs were cocultured with HUVECs or human intestinal FBs for 21 d. MC recovery was determined after 7, 14, and 21 d. (B) Percentage of BrdU$^+$ MC cultured for 7 and 14 d with cytokines (SCF, or SCF and IL-4) or with cells (HUVECs or FBs). (C) Measurement of sSCF in supernatants of HUVECs (either unstimulated or stimulated with 10 ng/ml TNF-α for 4 h) and of FBs. Means (± SD) of three experiments are shown.

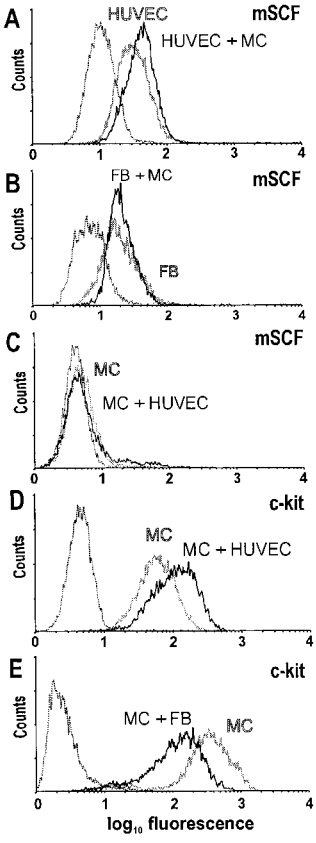

Figure 6. Expression of mSCF on HUVECs, FBs, and MCs, and expression of c-kit on MCs. (A) Expression of mSCF on HUVECs and (B) on human intestinal FBs with or without a 4-h coculture with MCs. (C) Expression of mSCF on MCs with or without a 4-h coculture with HUVECs. (D) Regulation of c-kit expression on the cell surface of MCs after a 4-h culture time with or without HUVECs. (E) Same as D, but HUVECs were replaced by human intestinal FBs. One representative experiment out of three is shown. In all histograms the leftmost peak (dotted line) represents the isotype control.

of c-kit expression on MCs was significantly increased (Fig. 6 D). In contrast, coculture of MCs with FBs clearly reduced c-kit expression on MCs (Fig. 6 E).

Adhesion Proteins Involved in MC–Endothelial Cell Interactions. We found by FACS® analysis that human intestinal MCs and FBs as well as HUVECs express a variety of adhesion molecules, summarized in Table I. With regard to selected adhesion molecules, the number of positive cells and mean relative fluorescence of stained cells were slightly modulated by coculture of the cells with other cell types (data not shown). The expressions of VCAM-1 and intracellular adhesion molecule (ICAM)-1 on HUVECs and FBs were enhanced after 4 h of coculture with MCs (Fig. 7 A). VCAM-1 expression on HUVECs cocultured with MCs increased in a time-dependent fashion and reached the maximum after 4 h. The upregulation of VCAM-1 was stable for up to 48 h (Fig. 7 B). MCs also increased E-selectin expression on HUVECs, whereas no E-selectin expres-

Table I. *FACS® Analysis of Adhesion Molecules on Human Intestinal MCs, Human Intestinal FBs, and HUVECs*

				MCs		HUVECs		FBs	
	Ab	Target	Ligands (examples)	% pc	Δmf	% pc	Δmf	% pc	Δmf
Selectins	CD62E	E-selectin	sLewis x, PSGL-1	25	3	30	7	0	0
	CD62L	L-selectin	sLewis x, PSGL-1	94	19	0	0	0	0
	CD62P	P-selectin	sLewis x, PSGL-1	28	2	0	0	0	0
Mucins	CD162	PSGL-1	P- and L-selectin	100	734	33	5	75	33
	CD15s	sLewis x	L-, E-, P-selectin	99	773	100	542	62	313
	CD43	Leucosialin	ICAM-1	100	436	0	0	0	0
β_1 integrins	CD29	β_1	–	100	273	100	2,804	97	2,241
	CD49a	α_1 (VLA-1)	Laminin	0	0	10	1	0	3
	CD49b	α_2 (VLA-2)	Collagen	97	23	100	860	100	392
	CD49c	α_3 (VLA-3)	Fibronectin	74	12	100	151	100	180
	CD49d	α_4 (VLA-4)	VCAM-1	100	106	100	57	98	227
	CD49e	α_5 (VLA-5)	Fibronectin	99	6	100	326	100	435
	CD49f	α_6 (VLA-6)	Laminin	0	0	91	149	0	0
β_2 integrins	CD18	β_2	–	88	24	0	0	0	0
	CD11a	LFA-1	ICAM-1, -2, -3	92	31	0	0	ND	ND
	CD11b	Mac-1	ICAM-1, -2, -3	17	1	0	0	ND	ND
	CD11c	P150, 95	C3bi, fibrinogen	55	4	0	0	ND	ND
β_3 integrins	CD61	β_3	–	100	56	99	157	72	322
	CD51	α_v	PECAM-1, vitronectin	66	13	97	32	62	37
β_7 integrins	–	β_7	–	60	22	23	4	30	24
	CD107a	$\alpha_4\beta_7$ (Act-1)	VCAM-1, fibronectin	36	5	58	15	50	25
Immunoglobulin superfamily	CD54	ICAM-1	LFA-1, Mac-1	99	104	59	16	89	470
	CD102	ICAM-2	LFA-1, Mac-1	33	4	100	1,244	17	6
	CD50	ICAM-3	LFA-1	42	4	0	0	0	0
	CD106	VCAM-1	VLA-4, LPAM-1	0	0	10	1	20	5
	CD31	PECAM-1	PECAM-1, LFA-1	43	5	100	957	0	0
	CD33	–	–	97	61	0	0	ND	ND
	CD63	–	–	100	81	100	70	100	501

FACS® results are expressed as percentage of positive cells (% pc) and difference in mean relative fluorescence between stained cells and isotype control (Δmf). One representative experiment (out of at least three) is shown. PECAM, platelet-endothelial cell adhesion molecule.

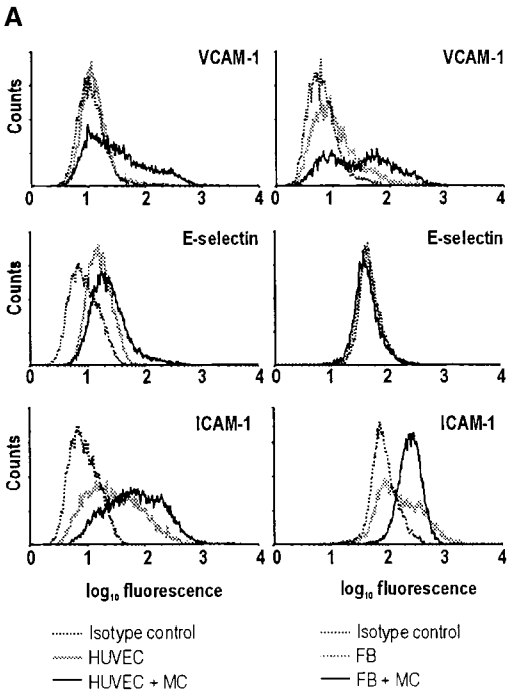

Figure 7. Regulation of VCAM-1, E-selectin, and ICAM-1 expression on HUVECs and FBs in coculture with MCs. (A) Expression of VCAM-1, E-selectin, and ICAM-1 on HUVECs (left panels) and human intestinal FBs (right panels) without (gray line) or with a 4-h coculture with MCs (black line). (B) Time-dependent expression of VCAM-1 on HUVECs (bars) and FBs (squares) after coculture with MCs. VCAM-1 expression on HUVECs and FBs is expressed as the difference between the geometric mean fluorescence of VCAM-1 staining and isotype control condition (Δmf). Basal VCAM-1 expression on HUVECs and on FBs not cocultured with MCs is shown in the leftmost bar (basal) or, respectively, leftmost square. One representative experiment out of three is shown.

sion was detectable on FBs (Fig. 7 A). FACS® analysis revealed that the inhibitory effect of TNF-α and IL-1β shown in Fig. 4 B is not related to the expression of VCAM-1 on HUVECs or VLA-4 on MCs (data not shown). Using the adhesion assay described previously, we tested different neutralizing Abs directed against adhesion molecules for their ability to inhibit MC adhesion to HUVECs and MC proliferation in coculture with HUVECs (Table II). MC adhesion to HUVECs was clearly reduced by the addition of Abs against CD29, CD49d, CD106, and CD62E. The simultaneous addition of mAbs against VCAM-1 and the two components of VLA-4 resulted in a reduction of MC adhesion to 26 ± 2% of the control ($P < 0.001$). Anti-SCF and anti–c-kit mAb also reduced significantly the adhesion of MCs to HUVECs (21 ± 5 and 23 ± 7%, $P < 0.001$), suggesting that at least two mechanisms are involved in MC–HUVEC interactions (SCF/c-kit and VCAM-1/VLA-4). The combination of all five neutralizing mAbs further reduced the adhesion of MCs to HUVECs (15 ± 3%, $P < 0.001$). The mAbs directed against adhesion molecules not only inhibited MC adhesion towards HUVECs but also HUVEC-dependent MC survival and proliferation. After 7 d of coculture, MC recovery was not yet changed significantly by addition of each neutralizing mAb or of all three mAbs together. However, after 14 d MC recovery was slightly reduced by each mAb, and a significant reduction of MC recovery (percentage of control, MC coculture with HUVEC and appropriate isotype controls) was observed if all three mAbs were administered simultaneously (17 ± 9%, $n = 3$; $P < 0.05$, Table II).

Table II. *Effect of Neutralizing Abs on MC Adhesion to HUVECs and MC Recovery in Coculture with HUVECs*

Ab	Target	Adhesion (percentage of control)	MC recovery (percentage of control)
Anti-CD29	β_1 integrin	35 ± 5	74 ± 34
Anti-CD49d	α_4 integrin	41 ± 9	86 ± 23
Anti-CD106	VCAM-1	39 ± 9	54 ± 42
Anti-SCF	SCF	21 ± 5	0
Anti–c-kit	c-kit	23 ± 7	0
Anti-SCF + Anti–c-kit	–	21 ± 7	0
Anti-CD29 + Anti-CD49d + Anti-CD106	–	26 ± 2	17 ± 9
Anti-CD29 + Anti-CD49d + Anti-CD106 + Anti-SCF	–	19 ± 4	0
Anti-CD29 + Anti-CD49d + Anti-CD106 + Anti-SCF + Anti–c-kit	–	15 ± 3	0
Anti-CD18	β_2 integrin	96 ± 3	ND
Anti-CD61	β_3 integrin	95 ± 2	ND
Anti-CD54	ICAM-1	70 ± 7	ND
Anti-CD62L	L-selectin	78 ± 13	ND
Anti-CD62E	E-selectin	52 ± 11	ND
Anti-CD62P	P-selectin	85 ± 4	ND

Means ± SD are shown, $n = 3$. Adhesion was determined after 4 h and MC recovery after 14 d. Controls were carried out with appropriate isotypes.

Discussion

In previous studies we and others found that isolated human MCs maintain in culture only if either the growth factor SCF was added or the cells were cocultured with mouse FB cell lines that express SCF (7, 22, 23). In this study we report for the first time that isolated human intestinal MCs survive for at least 21 d and proliferate in coculture with HUVECs without addition of any cytokines. Our preliminary results indicate that similar observations can be made if HUVECs are replaced by human endothelial cells isolated from intestinal tissue (data not shown), suggesting that the findings are not restricted to HUVECs.

According to our data, the effect of HUVECs on MCs resulting in increased MC numbers is because of enhanced MC proliferation and not because of decreased MC apoptosis. Surprisingly, human intestinal FBs were less potent in promoting MC survival and proliferation compared with endothelial cells. These data suggest that endothelial cells may be of particular relevance for the regulation of MCs in tissue in at least two means. Endothelial cells are clearly involved in the regulation of MC progenitor recruitment from the blood stream (24), an issue that was not addressed by these studies, and endothelial cells may regulate tissue MC density by modulating MC survival and proliferation, as indicated by our data.

Our results clearly indicate that membrane-bound molecules rather than soluble factors are mediating the effects of endothelial cells on MC survival and proliferation. According to our results, at least two molecular mechanisms are supposed to play a role, the SCF–c-kit and the VCAM-1–VLA-4 interaction, whereas IL-4 is obviously not involved. It is known from previous studies that HUVECs and endothelial cells of other origins, similar to FBs, express sSCF and mSCF (12, 13). We confirm these findings by showing that mSCF is expressed by HUVECs as well as intestinal FBs, and that FBs produce 17 times more sSCF than HUVECs. Inhibition studies using neutralizing Abs directed against SCF confirm the crucial role of mSCF in mediating endothelial cell–dependent MC survival and proliferation. The effects of HUVECs on MC survival and proliferation could by totally blocked by anti-SCF Ab. However, several functional studies indicate that SCF, which binds to c-kit expressed on MCs, may not be the only factor involved in this MC–endothelial cell interaction. For example, MC numbers were clearly higher in MC–HUVEC cocultures compared with MC cultures supplemented with SCF. Moreover, in 4 out of 13 experiments MCs died in the presence of sSCF, but not if co-cultured with HUVECs. The higher potency of HUVECs compared with SCF regarding MC survival is not related to IL-4, which was previously found to be capable of enhancing SCF effects (6), since neither MCs nor HUVECs expressed IL-4 in the coculture assay and HUVEC-dependent MC survival and proliferation was not affected by the addition of the competitive IL-4 inhibitor RY. Apart from IL-4, other cytokines known to have synergistic effects with SCF on MC proliferation may be involved in MC–endothelial cell interactions. However, at least for IL-3 and IL-5, we could largely exclude this possibility by using neutralizing Abs against IL-3 or its receptor, and by showing that IL-5 does not enhance SCF-dependent MC proliferation (our unpublished results). Thus, two other explanations may be envisioned. Either "solid phase SCF," such as mSCF, is more potent than sSCF with regard to MC proliferation, and/or yet unknown cofactors are involved in the SCF-dependent effects of endothelial cells on MCs. To address the second hypothesis, we studied several adhesion factors expressed on MCs or HUVECs because adhesion factors are candidates within membrane-bound factors that may modulate MC–endothelial interactions (25).

Human MCs of different origins express high levels of α_4 and β_1 integrin chains assembling to the VLA-4 complex that is also expressed on lymphocytes, eosinophils, and monocytes (26–28). VLA-4 binds to VCAM-1 expressed on endothelial cells and is involved in mediating adhesion of eosinophils, lymphocytes, and monocytes to the endothelium (29, 30). Similar results were obtained for the rat mucosal-type MC lines RBL-1 and RCMC-1 that express VLA-4 and bind to VCAM-1, suggesting a mechanism for MC accumulation at sites of inflammation (31). The high expression of α_4 and β_1 integrin chains on human intestinal MCs and the upregulation of VCAM-1 expression on HUVECs we found after coculture with MCs suggest a functional significance of this pathway. The facts that MC adhesion to the cell surface of HUVECs and that HUVEC-dependent MC proliferation was clearly inhibited by the addition of neutralizing mAbs directed against α_4, β_1, and VCAM-1 strongly support the notion that MCs and HUVECs interact through the VLA-4 complex and VCAM-1. This interaction may cause an enhancement of MC proliferation mediated by the binding of endothelial mSCF to c-kit on MCs. A synergism between the VCAM-1/VLA-4 and the mSCF/c-kit pathway may be anticipated and provides an explanation for the observation that HUVECs forming a direct cell–cell contact with MCs are more effective in mediating MC proliferation than soluble cytokines such as SCF.

In previous studies, FBs were found to provide MC survival in vitro (22, 23, 32, 33), whereas endothelial cells have not been examined in this respect. Both human and rodent MCs survived for different time periods (average 1–8 wk) if cultured on rodent FB cell lines (mostly 3T3 FBs derived from Balb/c mice or Swiss albino mice). The capacity of FB supernatants to provide MC survival was dependent on the amount of sSCF released by the different FB types (32). Accordingly, the effects of human intestinal FBs on MCs were comparable to those induced by SCF, but were clearly weaker than those of endothelial cells, despite the fact that FBs not only release sSCF but also express mSCF. Thus, the effect of FBs is most likely due to their release of SCF rather than their expression of mSCF. It remains unclear why human intestinal FBs are less potent in promoting MC survival and proliferation compared with endothelial cells, although they express both mSCF and VCAM-1 in similar amounts as HUVECs. This discrepancy may be related to our observation that HUVECs enhanced c-kit expression in MCs, whereas FBs downregulated it. Previous studies reported a downregulation of c-kit in human lung MCs and MC lines by SCF (34), which may be prevented by yet unknown endothelial factors. Alternatively, human intestinal FBs may produce factors that downregulate MC responsiveness towards mSCF and VCAM-1. This hypothesis is supported by our finding that a 4-h challenge of MCs with SCF or with Fcϵ receptor Ab, ionomycin, and cytokines such as IL-1β, IL-4, and TNF-α decreases c-kit expression on MCs (our unpublished results). Another possibility may be that HUVECs, but not FBs, express cofactors enhancing the effect mediated by

mSCF and VCAM-1. Such a cofactor may be E-selectin, which is expressed on HUVECs but not on FBs. Interestingly, the number of E-selectin–positive HUVECs is increased after coculture with MCs (from 30 to 61%), and the addition of anti-CD62E neutralizing mAb to the coculture clearly reduced MC adhesion to HUVECs, suggesting a functional significance of E-selectin in MC–HUVEC interactions.

Our finding that endothelial cells regulate human intestinal MC functions such as survival, proliferation, and possibly other functions not yet examined such as mediator release may have several clinical implications. Endothelial cells, in contrast to soluble growth factors, selectively support the survival and growth of so-called "connective tissue–type MCs" containing tryptase and chymase (MC_{TC}). Before coculture, only ~one third of the MCs are MC_{TC} (6), but after 14 d of coculture with HUVECs, more than four fifths of the MCs are of the MC_{TC} subtype, indicating that HUVECs rather selectively promote proliferation of MC_{TC} or induce a change in MC phenotype. The fact that endothelial cells preferentially support connective tissue–type MCs known to be involved in wound healing, tissue remodeling, and fibrosis suggests a function of MCs and endothelial cells in such processes. In contrast, sSCF and IL-4 support "mucosal-type MCs" (MC_T) thought to mediate inflammatory processes such as allergic reactions or inflammatory bowel disease (6, 7). Our observation that proinflammatory endothelial cell agonists such as IL-1β, TNF-α, and IL-4 reduced MC numbers in our coculture system suggests that endothelial cell–dependent MC_{TC} regulation is of particular importance during noninflammatory or postinflammatory conditions.

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