Coordinate regulation of Steel factor, its receptor (Kit), and cytoadhesion molecule (ICAM-1 and ELAM-1) mRNA expression in human vascular endothelial cells of differing origins*

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Abstract

Endothelial cells (EC) are a major component of the bone marrow and peripheral vasculature microenvironments and contribute to the regulation of hematopoiesis. Human EC cultured from umbilical vein (HUVEC) and adult aorta (HAEC) were compared to determine differences in levels of the multipotent cytokine, Steel factor (SLF), its receptor (Kit), intercellular adhesion molecule-1 (ICAM-1), and endothelial leukocyte adhesion molecule-1 (ELAM-1) before and after stimulation with human interleukin-1β (HL-1β), human tumor necrosis factor-α (hTNF-α), recombinant human (rh) SLF, phorbol 12-myristate 13-acetate (PMA), or calcium ionophore (A23187). HUVEC expressed four-fold higher basal levels of Kit and three-fold higher basal levels of SLF transcripts than HAEC. In contrast, the basal level of ICAM-1 mRNA was four-fold lower in HUVEC than in HAEC. These differences in expression persisted following activation. All five agonists downregulated Kit mRNA levels by 50 to 80%, but there remained a three-fold higher level of expression in HUVEC compared to HAEC. While SLF mRNA expression was increased four-fold by IL-1β or TNF-α and 50-fold by PMA or A23187, there was still a two-fold higher level in HUVEC than in HAEC. Similarly, production of cell-associated SLF was induced two-fold above basal level by PMA in HUVEC and HAEC, with HUVEC producing two-fold more than HAEC before and after stimulation. Production of soluble SLF was also increased six-fold in HUVEC and HAEC by PMA, but the HAEC produced slightly more than the HUVEC. Expression of ICAM-1 mRNA was increased 11-fold in activated HUVEC and HAEC, but the induced levels of both ICAM-1 and ELAM-1 mRNA were three-fold lower in HUVEC. The time course of SLF mRNA upregulation and Kit mRNA downregulation paralleled the upregulation of both cytoadhesion molecules. Differences between HUVEC and HAEC may be related to their vascular sources, but also suggest that disparate regulation of SLF, Kit, ICAM-1, and ELAM-1 expression could indicate a predisposition of neonatal EC toward impaired cytokine signal transmission.

Key words: Steel factor—Stem cell factor—Kit receptor—ICAM-1—ELAM-1

Introduction

Differentiation and proliferation of hematopoietic progenitor cells along specific developmental pathways is controlled by cytokine signals generated primarily from activated T lymphocytes, macrophages, endothelial cells, and fibroblasts [1]. We have previously demonstrated that the expression of GM-CSF [2], G-CSF, and IL-3 [3] is reduced in stimulated human peripheral blood mononuclear cells (MNC) from umbilical cord compared to adult cells. This immunological deficiency may contribute to the high incidence of neonatal cytopenia during states of increased demand [4], such as bacterial sepsis [5].

The recent discovery of a multipotent cytokine, known variously as mast cell growth factor [6], stem cell factor [7], Kit ligand [8], and Steel factor (SLF) [9], has led us to investigate its role in the dysregulation of neonatal hematopoiesis. SLF is a product of mesenchymal cells such as fibroblasts [6-8], fetal liver epithelial cells [6], spleen [7], and bone marrow stromal cells [6], comprising hematopoietic microenvironments known to be defective in Steel mutant mice [10]. A membrane-bound form of SLF [6,8] may promote intercellular contact with hematopoietic progenitor cells in the bone marrow [11,12], while the soluble form may facilitate homing to the marrow [13,14]. SLF acts synergistically with other cytokines to enhance proliferation [15,16] and colony formation [17,18] by human marrow progenitor cells [19,20] known to express the tyrosine kinase receptor for SLF [6-8], which is the product of the c-kit proto-oncogene [21] designated Kit. We have recently shown that SLF can also synergistically increase the response of neonatal rat neutrophils +, bacterial sepsis in combination with G-CSF [22].

EC make up 10 to 20% of the bone marrow stromal cell population in human long-term cultures [23], as well as lining the entire peripheral vasculature. Isolated human vascular EC are known to produce several hematopoietically active cytokines [1] and are capable of supporting granulopoiesis in vitro [24]. Their critical role in the regulation of hematopoiesis is becoming increasingly evident [25]. The interaction of leukocytes with EC during acute inflammatory response [26] and within the bone marrow [27] is mediated by several cytoadhesion molecules [28]. Neonatal neutrophils, however, are significantly impaired in their adherence to activated EC [29,30]. Direct contact between the progenitor cells and bone marrow stromal EC could facilitate localized transmission of cytokine signals [28]. EC are known to respond to inflammatory cytokines such as IL-1 and TNF-α, which are induced during sepsis [26], by accumulating transcripts of several myelopoietic cytokines [31] and cytoadhesion molecules [32,33].
Based on our previous observations of deficient cytokine expression in neonatal vs. adult MNC, we hypothesized that alteration of SLF expression may contribute to this dysregulation of neonatal hematopoiesis. Therefore, we have compared the basal and stimulated levels of SLF expression in cultured human EC from umbilical vein (HUVEC) and adult aorta (HAEC). Since the direct interaction of EC with blood cells could influence the effects of SLF, we have also compared the mRNA expression of two cytoadhesion molecules, ICAM-1 [34] and ELAM-1 [32], as well as that of the Kit receptor in HUVEC and HAEC.

Materials and methods

Culture of human EC
Proliferating cultures of HUVEC (second passage) and HAEC (fourth passage) were obtained from Clonetics Corp. (San Diego, CA). Cultures were maintained in endothelial cell growth medium (EGM), which contained 10 ng/ml epidermal growth factor, 1 µg/ml hydrocortisone, 12 µg/ml bovine brain extract, 1.6 U/ml heparin, and 2% fetal bovine serum (Clonetics). Immunofluorescent staining for Factor VIII–related antigen and acetylated low-density lipoprotein (Ac-LDL) (Organon Teknika, Rockville, MD) uptake was used to verify EC culture purity. Experiments were performed during the third through sixth passages when cultures reached approximately 80% confluence. While cell doubling times increased upon subsequent passages, expression of the molecules studied appeared to be unaffected. Three different HUVEC and four different HAEC isolates were also used, with similar results.

RNA isolation and hybridization
Cultures were stimulated with concentrations of agonists as follows: 30 ng/ml PMA (Sigma, St. Louis, MO), 10³ M A23187 (Sigma), 5 U/ml HIL-1β (C.W. Reynolds, BRMP-NCI, Frederick, MD), 100 U/ml hTNF-α (Boehringer Mannheim, Indianapolis, IN), and 50 ng/ml rhSLF (provided as a generous gift by Immunex, Seattle, WA). Total RNA was extracted using the guanidinium thiocyanate/phenol procedure [35]. RNA samples (5 to 10 µg) were subjected to electrophoresis on 1% agarose/formaldehyde denaturing gels [2] and transferred onto nylon membrane (Schleicher & Schuell, Keene, NH). RNA blots were hybridized sequentially with DNA probes, 32P-labeled using random oligonucleotide priming [36]. RNA transfer and probe removal were carried out as recommended by the membrane manufacturer. Templates for 32P-labeled probes were derived from human cDNA plasmid clones as follows: Kit, 540-bp EcoRI-HindIII fragment from pChkkit/17 (ATCC, Rockville, MD) [21]; SLF, 900-bp BamHI-HindIII fragment from pBS:MGF (provided as a generous gift by Drs. S.D. Lyman and S. Gillis, Immunex); ICAM-1, 900-bp pro I fragment from pG4H1.1ms (provided as a generous gift by Dr. T.A. Springer, Harvard Medical School, Cambridge, MA) [34]; ELAM-1, 1515-bp Xba I-HindIII fragment from pELAM-1 (provided as a generous gift by Dr. B. Seed, Harvard Medical School) [32]; and β-actin, 670-bp Rsa I fragment from pHH6A-1 [37].

Analysis of RNA blots
Transcripts were identified by comparison to published sizes with a 0.24-to-9.5-kb RNA Ladder (Gibco-BRL, Grand Island, NY) and use of human globulostoma cell lines A172 (Kit+) and U87 (SLF+, ICAM-1-) (ATCC). The Kit transcript was found to be 5.5 kb in length, corresponding to published values [21]. The primary SLF transcript was 6 kb, with less abundant transcripts of 3.5 and 2.5 kb, similar to mSLF mRNA [6-8]. The ELAM-1 transcript was 4 kb in length, in agreement with published values [32]. The primary transcript of ICAM-1 was 3.2 kb in size, with a less abundant transcript of 2.5 kb, as reported by Staunton et al. [34]. Relative signal strength of hybridized transcripts was estimated by two-dimensional densitometric scanning of autoradiograms using the ScanSys 5000 (USB, Cleveland, OH) and Bio-Image Model 505 (Millipore, Bedford MA) automated scanning systems. Values were normalized to β-actin hybridization, which was found to be representative of total RNA loading in these experiments. Comparisons were expressed as the mean difference between at least three pairs of data points.

ELISA analysis of hSLF expression
SLF levels from 30 ng/ml PMA-stimulated and unstimulated EC culture supernatants and whole-cell extracts were measured using a sandwich enzyme-linked immunosorbert assay (ELISA) method [38]. SLF added exogenously to the culture medium (200 ng/ml) and normal serum hSLF (3.1 ± 0.3 ng/ml in adults) [39] were used as positive controls for soluble SLF. The human globulostoma cell lines U87 and A172 were used as positive and negative controls for cell-associated SLF. Supernatant media were collected from EC cultures at the times indicated. Cultures were subjected to 0.025% trypsin/0.01% EDTA to release cells, which were then counted with a hemacytometer. Whole-cell extracts were prepared from 10 cm² cell monolayers by lysis on ice for 30 minutes with 300 µl RIPA buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 1 mM MgCl2; 0.5 mM PMSF; 1% Nonidet P-40; 0.5% Deoxycholate; 0.1% SDS; 20 U/ml DNase I). Cell extract total protein content was determined using the BCA protein assay (Pierce, Rockford, IL). Microtiter plates were coated with polyclonal rabbit anti-rhSLF Ig. Culture supernatants or cell extracts were added and incubated at 22°C overnight. Horse-radish peroxidase-conjugated mouse monoclonal anti-SLF Ab (7H6) was added and incubated for 2 hours at 22°C. Plates were washed and tetra-methyl benzidine was added as substrate. The reaction was stopped after 30 minutes by addition of 0.5 N sulfuric acid. Optical density was measured at 450 nm with an ELISA microplate reader (Bio-Rad, Richmond, CA).

Results

Basal mRNA levels of Kit, SLF, and ICAM-1
Basal transcript levels were compared in HUVEC and HAEC (Fig. 1). The HUVEC expressed four-fold higher basal levels of Kit mRNA than the HAEC (Fig. 1A). The basal SLF mRNA level was three-fold higher in the HUVEC than in the HAEC (Fig. 1B). In contrast, the basal level of ICAM-1 mRNA was four-fold lower in the HUVEC than the HAEC (Fig. 1C). Basal expression of ELAM-1 mRNA was undetectable. ICAM-1 mRNA expression was also greater in dermal fibroblasts from adults compared to newborns (data not shown).

Effect of agonists on Kit mRNA expression
II-1β maximally downregulated Kit mRNA expression by 50% after 2-hour stimulation in both HUVEC and HAEC (Fig. 2). Stimulation for 4 hours caused a 60% decrease with PMA or A23187, a 70% reduction with TNF-α, and an 80% decrease with rhSLF (Fig. 2). The Kit mRNA level returned to basal level after 24 hours with PMA (Fig. 3) or II-1β, but remained downregulated with A23187. There were no appreciable differences between the HUVEC and HAEC in the timing or degree of downregulation by these agonists. The level of Kit mRNA always remained approximately three-fold higher in the HUVEC during downregulation (Figs. 1A and 2).
**Fig. 1.** RNA blot analysis of unstimulated EC. Total RNA from unstimulated cultures of HUVEC (UV) and HAEC (AA), along with control cell lines A172 and U87, was prepared as described in Materials and Methods and hybridized to $^{32}$P-labeled probes for Kit and β-actin (A); SLF and β-actin (B); and ICAM-1 and β-actin (C). Results shown in A and B are representative of eight different experiments; those in C are representative of five different experiments.

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<tr>
<th>Control</th>
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**Fig. 2.** RNA blot analysis of Kit mRNA expression in stimulated EC. Cultures of HUVEC (UV) and HAEC (AA) were stimulated with PMA, A23187, TNF-α, or SLF for 4 hours each or IL-1β for 2 hours. Total RNA from these cultures was prepared as described in Materials and Methods and hybridized to $^{32}$P-labeled probes for Kit and β-actin, with corresponding bands indicated. Results shown are representative of five different experiments with PMA, four different experiments with IL-1β, and three different experiments with TNF-α, A23187, and SLF.

**Effect of agonists on SLF mRNA and protein expression**

IL-1β produced a maximum five-fold increase in SLF mRNA after 2-hour stimulation in both HUVEC and HAEC (Fig. 4). Although the SLF mRNA signal is not apparent in the AA control presented in Figure 4, the signal on the original autoradiogram was sufficient for densitometric quantitation.

Stimulation with PMA or A23187 for 4 hours resulted in a maximum 50-fold increase, while TNF-α caused only a three-fold increase after 4 hours (Fig. 4). rhSLF had no effect on SLF mRNA expression. Expression approached the basal level after 24 hours with PMA (Fig. 3), IL-1β, or A23187. As in the Kit response, there were no appreciable differences between
Fig. 3. Composite time course of Kit, SLF, and ICAM-1 mRNA expression after stimulation with PMA. HAEC cultures were stimulated with PMA for varying lengths of time as indicated. Total RNA from these cultures was prepared as described in Materials and Methods and hybridized to $^{32}$P-labeled probes for ICAM-1, SLF, Kit, and β-actin. Results of RNA blots were analyzed as described in Materials and Methods, and data are presented as the mean ± standard error of the mean (SEM) of the multiple difference from basal level in three different experiments. The basal level at 0 hours was defined as 1.0 for SLF, ICAM-1, and Kit in this graphic representation. HUVEC exhibited a similar time course, as described in text.

![Graph showing expression relative to basal level versus duration of PMA stimulation.](image)

Fig. 4. RNA blot analysis of SLF mRNA expression in stimulated EC. Cultures of HUVEC (UV) and HAEC (AA) were stimulated with PMA, A23187, or TNF-α for 4 hours each or IL-1β for 2 hours. Total RNA from these cultures was prepared as described in Materials and Methods and hybridized to $^{32}$P-labeled probes for SLF and β-actin, with corresponding bands indicated. Results shown are representative of five different experiments with PMA, four different experiments with IL-1β, and three different experiments with TNF-α and A23187.

![RNA blot images showing SLF and β-actin expression](image)

the HUVEC and HAEC in the timing or degree of SLF induction by these agonists, with the induced mRNA levels remaining approximately two-fold higher in the HUVEC (Figs. 1B and 4). The SLF protein was also present at 1.64 ng/mg protein in HUVEC and 0.73 ng/mg protein in HAEC extracts after 8-hour stimulation by PMA, which represented two-fold increases over the basal levels. This cell-associated SLF was two-fold higher in HUVEC than in HAEC before and after PMA stimulation (Fig. 5A). PMA also induced production of 1.8 and 1.5 ng soluble SLF/10⁶ cells in HUVEC and HAEC conditioned media after 24 hours, which was six-fold above the basal levels (Fig. 5B). Assuming complete extraction of cell-associated SLF, HUVEC produced a 2:1 ratio of soluble to cell-associated forms and HAEC produced a 5:1
ratio, while both expressed approximately equivalent amounts of total SLF per cell.

**Induction of ICAM-1 and ELAM-1 mRNA expression**

IL-1β stimulation for 2 hours induced a 12-fold increase in ICAM-1 mRNA, which persisted for 8 hours in both HUVEC and HAEC (Fig. 6). While the ICAM-1 mRNA signal is not apparent in the UV control presented in Figure 6, the signal on the original autoradiogram was sufficient for densitometric quantitation. PMA, A23187, or TNF-α produced a 12- to 16-fold increase after 4 hours (Fig. 6), which persisted through 8 hours with TNF-α and A23187, but diminished after 4 hours with PMA (Fig. 3). The induction of ELAM-1 could not be compared to a basal level, but the timing of its upregulation by these four agonists was equivalent to that of ICAM-1. rhSLF had no effect on the mRNA of either cytokinesis molecule. Expression of both transcripts returned to basal level after 8 hours with PMA (Fig. 3). Induction of these transcripts generally paralleled that of SLF in response to the agonists tested (Fig. 3). Similar to the above results for SLF and Kit expression, there were no appreciable differences between HUVEC and HAEC in timing or degree of response, with levels of ICAM-1 and ELAM-1 mRNA remaining threefold lower in the HUVEC after stimulation (Figs. 1 and 6).

**Discussion**

There was a higher level of Kit and SLF mRNA and cell-associated SLF expression by stimulated and unstimulated HUVEC compared to HAEC. The increased levels of SLF mRNA and cell-associated SLF in HUVEC contrast with our previous observations of decreased GM-CSF, G-CSF, and IL-3 expression in stimulated umbilical MNC [2,3]. Both SLF and the EC themselves, however, may play considerably different functional roles in the regulation of hematopoiesis. We have recently shown that post-bone marrow transplant associated neutropenia (≤200/mm³) did not inversely correlate with circulating SLF levels in contrast to a significant inverse correlation with circulating G-CSF levels [40]. This suggests the
involvement of diverse mechanisms in regulating expression of these cytokines. Alternatively, the changes in expression levels between HUVEC and HAEC could be related to the differing vascular sources of the EC. However, while differing in caliber and classification, the umbilical vein and adult aorta are functionally similar, conveying oxygenated blood into systemic circulation. EC from both sources were also found to be comparable in studies of morphological and biochemical markers [41,42].

The present study also demonstrated that HUVEC expressed lower mRNA levels for the cytoadhesion molecules that mediate their interaction with leukocytes. The decreased expression of these cytoadhesion molecule transcripts in HUVEC provides an intriguing parallel to the deficient expression of Mac-1 [29] and LECAM-1 [30], ligands for ICAM-1 [43] and ELAM-1 [44], respectively, in neonatal neutrophils. It has also been recently suggested that CD34 may play a role in inhibiting leukocyte adhesion and extravasation, since it is reciprocally downregulated by IL-1β or TNF-α while ICAM-1 and ELAM-1 are upregulated in HUVEC [45]. If Kit is performing a similar function, this inhibition would presumably be greater in HUVEC, where Kit mRNA expression is higher. It remains to be established if elevated Kit and reduced cytoadhesion molecule expression act to impair leukocyte adhesion with HUVEC, as compared to HAEC.

The ability of PMA and A23187 to reciprocally upregulate SLF, ICAM-1, and ELAM-1 and downregulate Kit mRNA suggested the involvement of a protein kinase C-dependent signal transduction pathway [46]. However, IL-1 [47] and TNF-α [48] signal pathways in fibroblasts do not appear to require protein kinase C activation or elevated cytosolic calcium. Thus, multiple distinct or overlapping pathways are likely to be involved in controlling expression of these transcripts as suggested previously in the cases of ICAM-1 [49] and GM-CSF [50] mRNA induction. This may account for the considerable differences in SLF mRNA levels induced by IL-1β or TNF-α compared to PMA or A23187.

The ability of rhSLF to downregulate Kit mRNA is supported by previous evidence for downregulation of the Kit receptor protein by soluble SLF [51]. This offers a possible mechanism for reciprocal downregulation of Kit mRNA upon upregulation of SLF by agonist stimulation. The similar levels of PMA-induced soluble SLF from HAEC and HUVEC support our previous demonstration of equivalent serum SLF levels in newborns and adults [39]. The differing ratios of soluble to cell-associated SLF produced by HUVEC compared to HAEC imply an alteration in the RNA splicing and/or proteolytic cleavage processes [14]. It is possible that increased HUVEC membrane-bound SLF may interfere with soluble SLF-dependent homing of circulating neonatal progenitor cells [4] to the bone marrow [13,14].

The interrelationship between expression of these four cell membrane-associated proteins may be an important component governing interactions of blood cells with the bone marrow and vascular microenvironments. In this in vitro study, we have demonstrated increased SLF and Kit but decreased ICAM-1 and ELAM-1 mRNA levels, along with increased cell-associated SLF in HUVEC vs. HAEC. Upregulation of SLF and downregulation of Kit transcripts paralleled the upregulation of ICAM-1 and ELAM-1 and was similar in stimulated HUVEC and HAEC. Since HUVEC and HAEC were derived from major blood vessels in the newborn and adult, we propose that differential expression of these molecules in vivo could affect transmission of cytokine signals and contribute to dysregulation of neonatal hematopoiesis. Comparisons of in vitro and in vivo expression by EC from
other sources, such as bone marrow, will be useful to test this hypothesis.

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