Constitutive Expression of Steel Factor Gene by Human Stromal Cells

By Michael C. Heinrich, Douglas C. Dooley, Alison C. Freed, Louise Band, Maureen E. Hoatlin, Winifred W. Keeble, Sandra T. Peters, Kirsten V. Silvey, Frederick S. Ey, David Kabat, Richard T. Maziarz, and Grover C. Bagby, Jr

Steel factor (SF), the ligand for c-kit, is an essential regulator of normal hematopoiesis, melanogenesis, gametogenesis, and mast-cell growth and development. Hematopoietic stromal cells are important sources of SF, because inactivation of SF in mice results in defects in the support function of hematopoietic stromal cells. To identify specific cells that produce, and factors that govern the expression of the different isoforms of SF in human hematopoiesis, we quantified levels of SF mRNA and membrane-bound protein in human stromal cells before and after exposure to recombinant human interleukin (IL)-1α, a cytokine known to induce the expression of a variety of hematopoietic growth factors. In addition, because stromal cells in long-term bone marrow cultures (LTBMC) are supportive of hematopoietic progenitor cell survival in vitro, while umbilical vein endothelial cells (EC) and diploid fibroblasts (DF) are not, we also sought to test the hypothesis that SF gene expression would differ in cells from LTBMC when compared with EC or DF. Using reverse transcription polymerase chain reaction amplification (RT-PCR), ribonuclease protection assays (RPA), and Northern blot analysis, SF was found to be constitutively transcribed in EC, DF, and LTBMC. IL-1α neither induced accumulation of SF mRNA nor altered the ratio of exon 6+ to exon 6− transcripts in these stromal cells. By Northern blot analysis, the predominant SF mRNA species was shown to be 5.6 kb; a minor population of 3.6 kb was also found. Low levels of membrane-bound SF protein were found to be constitutively expressed by all three types of stromal cells, and were not regulated by IL-1α. We conclude that the unique capacity of LTBMC to support in vitro hematopoiesis, when compared with EC or DF, cannot be explained on the basis of qualitative or quantitative differences in SF gene expression in these cells.

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variety of hematopoietic defects, including macrocytic anemia. In addition, these mice are sterile, have reduced numbers of skin mast cells, and lack pigment, except in the retina. Membrane-bound SF protein stimulates the proliferation of c-kit receptor–bearing cells, and may also mediate cell-cell adhesion. For these reasons, the numbers of skin mast cells, and lack pigment, except in the retina.

Although endothelial cells and fibroblasts stimulated by IL-1 can support hematopoiesis by expressing multilineage hematopoietic growth factors, uninduced endothelial cell and fibroblast monolayers are less capable of supporting hematopoietic progenitor cell growth and differentiation than stromal cells in LTBMC. We therefore sought to test two hypotheses: (1) that IL-1 can induce the accumulation of SF mRNA in stromal cells and thereby increase the production of SF protein by these cells; and (2) that the exon 6+SF transcript, which is translated to produce a predominantly membrane-bound SF protein, is more prevalent in LTBMC than in endothelial cells or fibroblasts, resulting in increased expression of the membrane-bound SF protein isoform.

SF mRNA expression in human umbilical vein endothelial cells (EC), normal diploid fibroblast (DF), and LTBMC was determined using reverse-transcription polymerase chain reaction amplification (RT-PCR), ribonuclease protection assays (RPA), and Northern blot analysis. E-selectin mRNA was used as a positive (IL-1-inducible) control. SF transcript is constitutively transcribed in EC, DF, and LTBMC, and is not inducible by IL-1α. The ratio of exon 6+SF transcripts to exon 6−transcripts was identical in the three stromal cell types that were analyzed, and was not altered by treatment of these cells with IL-1α. Membrane-bound SF was assessed using fluorescent cell staining. The leukocyte adhesion molecules ICAM-I and E-selectin were used as positive (IL-1-inducible) controls. Membrane-bound SF was present on all three types of stromal cells and was not increased by treatment of these cells with IL-1α.

### MATERIALS AND METHODS

#### Reagents

Recombinant human IL-1α (3 × 10⁶ U/mg) was generously provided by Dr Peter Lomedico (Hoffman-LaRoche, Nutley, NJ). IL-1α–treated cells were exposed to 2.5 ng/mL of IL-1α for 3 to 72 hours. RR1/1 monoclonal antibody (MoAb) was used to detect the CD54 antigen (ICAM-1), a gift from Dr Timothy Springer, Harvard Medical School, Boston, MA. MoAb 12G11.1 was used to detect E-selectin (a gift from Dr Tom Thiemer, Epitope, Beaverton, OR). Purified MoAb supernatant (anti–H-2Dk) was prepared from the 15-5-5 SS hybridoma cell line (ATCC HB24; ATCC, Rockville, MD) and was used as a negative control antibody. Polyclonal rabbit serum was obtained from rabbits immunized with recombinant human SF (HuMFG-P1; a gift from Dr Douglas Williams, Immunex, Seattle, WA). Affinity-purified polyclonal goat IgG specific for human SF was obtained from R & D Systems (Minneapolis, MN). Normal rabbit serum and fluorescein isothiocyanate (FITC)-conjugated affinity-purified polyclonal rabbit anti-goat IgG antibody were purchased from Sigma (St Louis, MO). FITC-conjugated goat antimouse antibody and goat anti-rabbit antibody were purchased from AMAC (Westbrook, ME).

#### Plasmids

pGEM-4Z E-selectin. A 1,074-bp fragment (bases 562 to 1,635) of E-selectin (formerly designated ELAM-1) was isolated following digestion of H3M ELAM-1 with PstI restriction enzyme. This fragment was cloned into a PstI-digested pGEM-4Z plasmid (Promega, Madison, WI) following manufacturer’s directions. The H3M ELAM-1 plasmid was a generous gift of Dr Michael Bevilacqua (University of California at San Diego School of Medicine, San Diego, CA).

pSP72 GAPDH. pHcGAP was sequentially digested with PstI and Xbal restriction enzymes to release a 780-bp fragment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was subsequently cloned into a pSP72 plasmid (Promega). pHcGAP contains the cDNA for GAPDH as described by Wu et al, and was obtained from ATCC (plasmid no. 57090, ATCC).

pHuMGF 2.4. This plasmid has a 900-bp insert representing the entire human SF coding sequence (bases 160 to 1,053 using the notation system of Martin et al). This plasmid was the generous gift of Dr Stewart Lyman (Immunex).

#### Table 1. PCR Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Location</th>
<th>Exon</th>
<th>Strand</th>
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<td>696-716</td>
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<td>antisense</td>
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Underlined bases indicate synthetic restriction enzyme recognition sites included to allow cloning of the amplification product.
pGEM3 γ-actin. A 700-bp HindIII-BamHI fragment of human γ-actin isolated from plasmid pHFγA-3′UT was cloned into a pGEM-3 plasmid (Promega). The pHFγA-3′UT plasmid contains a partial fragment of human fibroblast cytoplasmic γ-actin 3′ untranslated region (UTR), and was the generous gift of Dr Peter Gunning (Stanford University School of Medicine, Palo Alto, CA).32,33

pGEM-4Z SF. Using primers S-1 and S-2 (Table 1), a 721-bp fragment of human SF was generated by RT-PCR amplification of EC RNA. Following digestion of the RT-PCR product with BamHI and XhoI restriction enzymes, the DNA was cloned into a BamHI/XhoI-digested pGEM-4Z plasmid.

pSSF. This plasmid encodes a colinear molecular clone of Friend spleen focus-forming virus and has been previously described.31,32

pSSF MGF. pHu MGF 2.4 was linearized with BamHI, blunt-ended with Klenow PolI fragment, and further digested with XhoI to release the SF fragment (bases 160 to 1053 using the notation system of Martin et al).35 The SF fragment was ligated into a pSFF vector, which had been linearized with EcoRI, then blunt-ended with Klenow PolI fragment, and further digested with XhoI.

Cell Lines

EC, used between fourth and tenth passage, were cultured as described previously.24 IL-1 stimulation/experiments were performed using media that did not contain EC growth factor. Human lung-derived DF (CCD-11Lu, ATCC no. CCL 202) were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Ronkonkoma, NY) and maintained in RPMI supplemented with 10% fetal bovine serum.38 $-2 ecotropic-packaging cells39 and PA12 cells were provided by Dr Robert Handin (Brigham and Women's Hospital, Boston, MA). Neonatal foreskin fibroblasts (NFF) were isolated as described previously,40 and grown in DMEM supplemented with 10% fetal bovine serum. JY, a B-lymphoblastoid cell line, was maintained in RPMI supplemented with 10% fetal bovine serum.24 Dami, a megakaryoblastic leukemia cell line, was generously provided by Dr Robert Handin (Brigham and Women’s Hospital, Boston, MA) and maintained in RPMI supplemented with 10% fetal bovine serum.24,41-43 $-2 cotropic-packaging cells44 and PA12 cells amphotrophic-packaging cells45 were grown in DMEM supplemented with 10% fetal bovine serum.24 Bone marrow was obtained from normal donors at the time of collection for allogeneic transplantation. Following Ficoll-Hypaque (Pharmacia, Piscataway, NJ) separation, low-density cells were seeded into T-175 flasks (1 x 10⁶ cells per flask) containing 50% deionized formamide, 5X Denhardt's, 5X SSPE, 0.1% SDS at room temperature for 30 minutes. After 7 days at 37°C, cultures were fed by replacement of half of the medium. Subsequently, cultures received complete media changes at weekly intervals. When cultures reached near confluence (3 to 4 weeks), they were subsequently maintained by trypsinization and subculturing at 2:1 to 4:1 ratios. Cells were analyzed between second and fourth passage. When maintained in this fashion, the stromal layers were fully capable of supporting committed progenitors and long-term culture-initiating cells.43

Retroviral Vector Amplifications

Purified pSFF or pSSF MGF plasmid DNA (10 μg) was transfected as a calcium phosphate precipitate, using protocol A as previously described, into culture dishes that contained a 1:1 mixture of φ-2 and PA12 cells.44

RNA Isolation

Total RNA was prepared using the method of Cathala et al.45 Cells were lysed in 5 mol/L guanidine isothiocyanate (GTC; Fluka, Ronkonkoma, NY) and the RNA precipitated using 4 mol/L LiCl. Following precipitation, the pellets were digested with 150 μg/mL proteinase K (Sigma). RNA was purified from the digested, solubilized pellets by repeated phenol/chloroform/isoamyl alcohol extractions, followed by two ethanol precipitation steps.

RT-PCR

A 20-μL reverse-transcription mixture containing 2.5 μg of RNA in 1X PCR buffer (10 mmol/L Tris HCl [pH 8.3], 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.1% gelatin), 1 mmol/L dithiothreitol, 2.5 mmol/L MgCl₂ (total MgCl₂, 4 mmol/L), 1 mmol/L dNTPs (Pharmacia), 40 U RNasin (Promega), 0.2 μg random hexamer primers (GIBCO BRL), and 1 μL of RNase H- MMLV reverse transcriptase (GIBCO BRL) was incubated for 60 minutes at 37°C. The reverse-transcriptase enzyme was inactivated by heating the reaction mixture to 95°C for 5 minutes. The cDNA was diluted 10-fold, and 1/40 (5 μL) of each reaction was used for PCR as described by Saiki et al.46 Each 100-μL PCR reaction contained 100 pmol of each primer, 2.5 μL of Taq polymerase (AmpliTaq; Perkin Elmer-Cetus, Norwalk, CT), 200 μmol/L of each dNTP, 10 μL of 1X PCR buffer, and 5 μL of diluted cDNA. The reactions were amplified in a Coy Model 50 Temp Cycler (Coy Laboratories, Ann Arbor, MI). One PCR cycle consisted of 1 minute at 94°C, 1 minute at 50°C, and 1 minute at 72°C. Typically, 25 to 35 cycles of amplification were performed. Table 1 lists the nucleotide sequence and cDNA location of the PCR primers used in our studies.

Southern Blot Analysis

PCR products were size-fractionated by gel electrophoresis through 1% to 1.5% agarose, and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). Nucleic acids were immobilized by UV irradiation (Stratalinker; Stratagene, La Jolla, CA). Prehybridization was performed for 2 hours at 42°C in a solution containing 50% deionized formamide, 5X Denhardt's, 5X SSPE (5X SSPE = 0.15 mol/L NaCl, 0.01 mol/L NaH₂PO₄, 0.001 mol/L EDTA), 100 μg/mL sheared salmon sperm DNA, and 0.5% sodium dodecyl sulfate (SDS). Hybridization was performed for 12 to 16 hours at 42°C in prehybridization solution containing 1 x 10⁶ cpm/μL of α-32P-γ-adenosine triphosphate (ATP) end-labeled oligonucleotide probe48 or random hexamer primed SF cDNA. The membranes were washed in 2X SSC (1X SSC = 0.15 mol/L NaCl + 0.01, mol/L sodium citrate)/0.1% SDS at room temperature for 30 minutes and then in 0.1X SSC/0.1% SDS at 50°C for 30 minutes.

Quantitative RT-PCR

Serial dilutions of gel-purified SF or E-selectin plasmid-derived cDNA, typically 1 pg to 10 ng, were amplified by PCR to generate a standard curve. PCR cycle number was optimized to maintain exponential conditions of amplification. Fifteen-microliter aliquots of the reaction were analyzed by Southern blot technique. Densitometry was performed using a Bio-Rad Model 620 one-dimensional (1-D) densitometer (Bio-Rad, Rockville Centre, NY).

Least-squares regression was used to fit the densitometry data to the equation: Optical density = slope x [gplasmid cDNA in picograms] + intercept. The regession equation was used to predict the relative amount of SF or E-selectin cDNA in simultaneously
amplified and Southern blotted samples of reverse-transcribed EC RNA.

RPA

RPA were performed as described previously using a commercially available kit (RPA II; Ambion, Austin, TX).49 In vitro antisense RNA transcripts were uniformly labeled with $^{32}$P-$\alpha$-uridine triphosphate (UTP). T3 RNA polymerase was used to generate SF antisense RNA transcripts from Ncol-digested pHuMGF 2.4. T7 RNA polymerase was used to generate SF antisense RNA transcripts from EcoRI-digested pGEM-4Z SF. T7 RNA polymerase was used to generate E-selectin antisense RNA transcripts from BamHI-digested pGEM-4Z E-selectin, and GAPDH antisense transcripts from Ncol-digested pSP72 GAPDH. For each experimental condition, 5 to 10 pg of RNA was hybridized with I X 10⁵ cpm of antisense probe for 16 hours at 45°C in the presence of 80% deionized formamide, 40 mmol/L PIPES (pH 6.4), 400 mmol/L sodium acetate (pH 6.4), and 1 mmol/L EDTA. Following hybridization, the samples were digested with RNase A and RNase T1 for 1 hour at 37°C. The concentration of RNase was optimized for each antisense probe used. SF antisense transcript samples were digested with 1.67 U/mL of RNase A and 333 U/mL of RNase T1.

Northern Blot Analysis

RNA was size-fractionated on 1% agarose formaldehyde gels and transferred to nitrocellulose using 10× SSPE as a transfer buffer. Following transfer, the nucleic acids were immobilized by UV irradiation. Membranes were prehybridized, hybridized, and washed as described for Southern blots.24

Indirect Immunofluorescence

Before staining, adherent cell lines were released from culture flasks by incubation with trypsin-EDTA (GIBCO-BRL). Cells were washed in DMEM with 0.02% sodium azide. Harvested cells were incubated with MoAb, rabbit serum, or goat IgG and stained using an FITC-conjugated species-specific antiimmunoglobulin antibody. Surface-stained cells were fixed in 2% paraformaldehyde.
and analyzed by flow cytometry using a FACScan instrument (Becton Dickinson, Mountain View, CA). Trypsin-EDTA treatment of cells was shown not to alter indirect immunofluorescent detection of SF, ICAM-1, or E-selectin (data not shown).

RESULTS

EC Express SF mRNA

SF, GAPDH, and E-selectin mRNA from control and IL-1α-stimulated EC were analyzed by RT-PCR (Fig 1). SF transcripts were detectable in equal amounts in both control and IL-1α-stimulated EC. In contrast, there was at least 50-fold more E-selectin RT-PCR product obtained from IL-1-treated EC RNA than from control EC RNA. A similar pattern of induction was seen in RT-PCR reactions using granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6 primers (data not shown). GAPDH transcript was present in equal amounts in control or IL-1α-treated EC RNA than from control EC RNA. A similar pattern was observed when GAPDH transcripts were amplified to generate a 257-bp product, whereas E-selectin transcripts were equivalent in control and IL-1α-stimulated EC RNA (data not shown).

Expression of SF by Fibroblasts and LTBMC Adherent Cells

We and others have previously noted similar patterns of expression of hematopoietic growth factors by EC, DF, and LTBMC. Therefore, RPA and RT-PCR analyses of RNA prepared from DF and LTBMC were performed to determine whether these cell types also expressed constitutive SF transcript. The concentration of SF transcript was similar in these three stromal cell types (Fig 4). As was the case for EC, no significant effect of IL-1α on DF or LTBMC expression of SF transcript was found.

Alternatively Spliced SF mRNAs

Our previous RT-PCR studies of SF transcripts used an antisense primer (S-4; Table 1) complementary to exon 6, thereby amplifying only exon 6+ SF transcripts. To discriminate between exon 6+ and exon 6- transcripts, RT-PCR of stromal cell RNA was performed using a primer pair (S-6 and S-7; Table 1) whose amplification product spanned exons 4 through 8. Using this primer pair, exon 6+ transcripts were amplified to generate a 757-bp product, whereas exon 6- transcripts were amplified to yield a 673-bp fragment. These amplification products were resolved by 1.5% agarose gel electrophoresis, and the relative product amounts determined by Southern blot analysis. Both species of SF transcript were constitutively expressed in EC, DF, and LTBMC (Fig 5). In all three stromal cell types studied, there was a predominance of exon 6+ transcripts, and no significant difference was found in the ratio of exon 6+ to exon 6- transcripts between the three types of stromal...
Northern Blot Analysis of SF Transcripts

Northern blot analyses were performed to allow measurement of SF transcript size. At least two species of SF transcript were found expressed in EC (Fig 6A). The most prevalent SF transcript had an estimated size of 5.6 kb. The smaller transcript, which accounts for only 15% of total SF mRNA detected, had an estimated size of approximately 3.6 kb. No effect of IL-1α on SF transcript amount was seen by Northern blot analysis. In contrast, E-selectin mRNA accumulated substantially after IL-1α stimulation (Fig 6B). Both of these SF transcripts were also present in DF (Fig 7).

“Ping-Pong” Amplification of Retroviral Vector Containing Human SF cDNA

Because there are no standard methods for quantification of SF, we sought to validate our immunofluorescence approach using cells expressing high levels of human SF. To accomplish this, we developed a retrovirus that contained the human full-length SF cDNA. Retroviral vectors become efficiently amplified after they are transfected into cocultures of cells (eg, ψ-2 and PA12 fibroblasts) that package retroviruses into distinct host-range envelopes.35,56,57 The ψ-2 cell line releases a virus with an ecotropic host range, whereas the PA12 packaging cell line releases virions with an amphotropic host range.40 Since the virions from either cell type are cross-infectious,57,58 a back-and-forth (“ping-pong”) process of infectious amplification ensues until a large proportion of the cells express virally derived proteins. Insertion of a gene of interest into the retroviral vector results in a high level of expression of recombinant protein by cells in this coculture system. Ping-pong amplification has been previously used to achieve high-level protein production of human growth hormone,44 as well as murine erythropoietin.55,59 We used the ping-pong amplification system to induce expression of human SF by cells that were initially devoid of human SF protein (murine retroviral packaging cell lines ψ2 and PA12).

Amplified cocultures transfected with pSFF (ψ MOCK) or pSFF MGF (ψ SF), were analyzed for SF protein expression by indirect immunofluorescence using rabbit or goat antisera to human SF. As shown in Fig 8, ψ MOCK cells had no detectable membrane-bound human SF protein, whereas ψ SF cells expressed high levels of membrane-bound SF protein. Similar results were obtained using purified polyclonal goat antihuman SF IgG (data not shown). Expression of human SF mRNA by ψ SF, but not by ψ MOCK cells, was confirmed by Northern blot analysis (data not shown).

To confirm the specificity of our immunofluorescence assay for SF protein, we analyzed three lymphohematopoietic cell lines (Dami, JY, and HUT-78), which were found to lack detectable SF transcript in an RT-PCR assay. There

Table 3. Comparison of RPA and RT-PCR Estimates of EC E-Selectin mRNA (expressed as percent of maximal concentration)

<table>
<thead>
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<th>RNA</th>
<th>RPA (%)</th>
<th>RT-PCR (%)</th>
<th>Value</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.8% ± 4.0% (n = 10)</td>
<td>1.0% ± 0.3% (n = 7)</td>
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<tr>
<td>IL-1α, 3 hours</td>
<td>100.0% ± 18.2% (n = 10)</td>
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<td>IL-1α, 6 hours</td>
<td>85.1% ± 29.9% (n = 10)</td>
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<tr>
<td>IL-1α, 24 hours</td>
<td>22.5% ± 18.9% (n = 8)</td>
<td>11.8% ± 7.4% (n = 7)</td>
<td>17</td>
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</table>

Values (mean ± SD) are expressed as percent of maximal E-selectin mRNA concentration in control and IL-1α (2.5 ng/mL)-stimulated EC. A two-tailed Student's t-test was used to test statistical significance.

Table 4. Effect of Prolonged Incubation of IL-1α on EC SF mRNA Concentration

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<th>RNA</th>
<th>SF mRNA (%)</th>
<th>P Value</th>
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<tr>
<td>EC control</td>
<td>98.3% ± 32.1 (n = 4)</td>
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<td>EC IL-1α, 24 hours</td>
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<td>EC IL-1α, 72 hours</td>
<td>84.7% ± 40.6 (n = 4)</td>
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SF mRNA concentration was measured by RPA or Northern blot analysis. Values (mean ± SD) are expressed as percent of maximal SF mRNA concentration in control and IL-1α (2.5 ng/mL)-stimulated EC. A two-tailed Student's t-test was used to test statistical significance. For statistical testing, the mean SF mRNA concentration of each experimental condition was compared with the SF mRNA concentration in control EC.
Fig 4. Expression of SF mRNA in EC, DF, and LTBMC. RNA was isolated from control or IL-1α-treated EC, DF, and LTBMC. EC and LTBMC were treated with IL-1α for 3 hours, DF were treated for 12 hours. RT-PCR was performed using primers S-3 and S-4 (Table 1). Following Southern transfer, the membrane was probed with 32p-γ-ATP end-labeled primer S-5.

Fig 5. Expression of exon 6+ and exon 6- SF transcripts in stromal cells. RNA was isolated from control or IL-1α–treated EC (0, 3, 6, and 24 hours), DF (6 hours), or LTBMC (4 hours). RT-PCR was performed using primers S-6 and S-7 (Table 1). Using this primer pair, exon 6+-containing transcripts were amplified to generate a 757-bp product, whereas exon 6- transcripts were amplified to yield a 673-bp fragment. Following Southern transfer, the membrane was probed with a random hexamer-primed BamHI/HindIII SF cDNA fragment isolated from pHuMGF 2.4.

was no difference in immunofluorescence of these cells when stained with either goat- or rabbit-produced anti-SF antibody or with control antibody (data not shown).

Constitutive Expression of Membrane-Bound SF Protein by Stromal Cells

EC, DF, and LTBMC were analyzed for the presence of membrane-bound SF using indirect immunofluorescence as described above. All three types of stromal cells express low but reproducibly detectable amounts of membrane-bound SF protein (Fig 8). Higher levels of membrane-bound SF protein were found in NFF and other skin-derived fibroblasts than in lung-derived fibroblasts (DF), EC, or LTBMC (Fig 8, and data not shown). Similar results were obtained using purified polyclonal goat anti-human SF IgG (data not shown).

IL-1α Does Not Alter SF Membrane-Bound Protein Expression by Stromal Cells

To determine whether IL-1 treatment would increase SF membrane-bound protein expression, we treated EC and NFF with IL-1α for 0 to 48 hours. Levels of SF protein and the leukocyte adhesion molecules ICAM-1 and E-selectin were determined by immunofluorescence (Fig 9). SF membrane-bound protein does not increase following treatment of EC with IL-1α. In contrast, E-selectin protein is undetectable in unstimulated EC, but treatment of EC with IL-1α resulted in a marked increase in E-selectin protein at 4 hours, with a return to nearly undetectable levels at 24 and 48 hours (Fig 9, and data not shown). ICAM-1 protein is present in low but detectable amounts in unstimulated EC, and treatment of EC with IL-1α resulted in an increase in ICAM-1 protein after only 4 hours, with a greater induction seen at 24 and 48 hours. SF membrane-bound protein was not increased by treatment of NFF with IL-1α. E-selectin protein is not expressed by fibroblasts, but the time course of IL-1 induction of ICAM-1 protein in NFF was the same as in EC. Similar results were obtained with lung-derived fibroblasts (data not shown).

DISCUSSION

Using three separate methods, we have determined that EC, DF, and LTBMC constitutively transcribe the SF gene and express membrane-bound SF protein. IL-1α, known to induce the expression of a variety of cytokines, interleukins, hematopoietic growth factors, and adhesion molecules including IL-1, IL-6, IL-8, GM-CSF, G-CSF, E-selectin, VCAM-1, and ICAM-1 does not induce SF mRNA accumulation. Soluble SF protein synergizes with G-CSF, GM-CSF, IL-1, IL-3, and IL-6 in vitro hematopoietic cell colony growth assays. Therefore, the constitutive expression of SF by stromal cells likely contributes to the ability of these cells to support hematopoiesis not only in the steady-state, but also during conditions of inflammatory stress.

The c-kit and c-fms gene products are members of the receptor tyrosine kinase family, and display a high degree of structural and genetic homology. The ligands for these receptors, SF and M-CSF, respectively, enhance cellular growth and/or differentiation. Binding of cognate ligands
Table 5. Comparison of Exon 6+ to Exon 6- SF Transcripts in Human Stromal Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Ratio of Exon 6+ to Exon 6- SF mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC</td>
<td>3.50 ± 1.58 (n = 20)</td>
</tr>
<tr>
<td>LTBMC</td>
<td>2.90 ± 1.34 (n = 12)</td>
</tr>
<tr>
<td>DF</td>
<td>3.52 ± 1.58 (n = 12)</td>
</tr>
</tbody>
</table>

RT-PCR was performed with primers S-6 and S-7 (Table 1) using RNA from unstimulated EC, DF, or LTBMC. Following Southern transfer, the membranes were probed with a random hexamer-labeled SF cDNA (BamHI/HindIII fragment from pHuMGF 2.4). Following autoradiography, 1-D densitometry was used to determine the amount of exon 6+ (757 bp) and exon 6- (673 bp) amplification products. Data are expressed as the ratio of exon 6+ RT-PCR product to exon 6- RT-PCR product. Values represent the mean ± SD.

to the c-kit or c-fms receptor may activate similar signal transduction pathways, as the ectopic expression of the c-fms protein in c-kit mutant (W phenotype) murine mast cells can complement the mutation-induced defects in mast-cell function.73

Although there is little primary sequence amino acid homology between M-CSF and SF,13 the predicted protein tertiary structure and spatial arrangement of the functional domains of these molecules suggests a common structural framework that may account for the similarities in the biology of these growth factors. In both the M-CSF and the SF genes, exons 2 through 5 encode a cytokine domain helix-loop framework, exon 6 encodes a proline-serine-rich variable spacer chain that contains the preferred proteolytic cleavage site, and exon 7 encodes a tether chain that includes the transmembrane segment.74

Both M-CSF and SF nuclear RNAs are differentially spliced to give rise to different isoforms.14,44,75 The M-CSF exon 6 spacer chain sequence can be removed during splicing (M-CSFα transcript), or be spliced in and translated to yield a 298- or 116-amino acid spacer chain (M-CSFβ and M-CSFγ transcripts, respectively).72,74 All three M-CSF transcripts are translated to yield different-sized membrane-bound M-CSF proteins, which may be proteolytically cleaved to produce biologically active soluble M-CSF.76,77 The protein translated from the 4-kb M-CSFβ transcript undergoes rapid proteolytic cleavage and thus exists almost exclusively as a soluble protein. In contrast, the protein translated from M-CSFα mRNA is cleaved slowly and exists predominantly as a membrane-bound molecule.77 M-CSF, like SF, is biologically active as both a soluble and a membrane-bound protein.13,18,78 The proteolytic cleavage of M-CSF membrane-bound protein has been shown to be accelerated by treatment of M-CSF expressing cells with phorbol esters in a similar fashion as that of cells that express SF protein.19,79 As is the case with M-CSF, translation of alternatively spliced SF mRNA has been reported to produce either a predominately membrane-bound (from exon 6- transcripts) or predominately soluble (from exon 6+ transcripts) SF protein.13,14,18 Our experiments confirmed the constitutive expression of both species of SF transcript in EC, DF, and LTBMC.

“Competitive” RT-PCR provides an accurate determination of the initial ratio of PCR target sequences when the predominant species is present in less than a 10-fold molar excess and when PCR conditions allow exponential amplification.80 Using primers S-6 and S-7 (Table 1), the ratio of exon 6+ and exon 6- SF transcripts was determined using

Fig 6. Northern blot analysis of EC RNA. RNA was isolated from EC exposed to either control medium or IL-1α for 4 hours. Twelve micrograms of RNA from each condition was analyzed by Northern blot. (A) SF mRNA. The membrane was hybridized with a random hexamer-primed BamHI/HindIII fragment of SF cDNA isolated from pHuMGF 2.4. The predominant species of SF has a molecular weight of 5,600 bases, and the minor species has a MW of 3,600 bases. (B) E-selectin mRNA. Following hybridization with the SF cDNA probe, the membrane was stripped and rehybridized with a PstI/PstI fragment of E-selectin cDNA isolated from pGEM-4Z E-selectin. (C) γ-Actin mRNA. Following hybridization with the E-selectin probe, the membrane was stripped and hybridized with a PstI/BamHI fragment of γ-actin cDNA isolated from pGEM3 γ-actin.
EC DF

5600 bp - SF

3600 bp -

competitive RT-PCR. All three types of stromal cells contained both SF transcript species, and had similar exon 6 to exon 6' ratios of approximately 3:1. The ratios obtained from our RT-PCR and RPA studies are similar to those obtained using RPA to analyze SF splicing in various murine and human tissues.\textsuperscript{14,19,84}

There are at least two species of SF transcripts detectable by Northern blot analysis of EC and DF RNA. The larger species has a size of approximately 5.6 kb, which is consistent with the estimates obtained from analysis of the human SF cDNA sequence and by previous Northern blot analyses.\textsuperscript{17} The alternative usage of exon 6 during SF splicing does not explain the presence of two SF transcripts detected by Northern blot studies, as exon 6 contains only 84 bp, and exclusion of only exon 6 during splicing would not produce a SF transcript almost 2 kb smaller than the predominant SF transcript. M-CSF transcripts that use different 3' non-coding sequence exons have been described.\textsuperscript{85} Although it is possible that the smaller 3.6-kb transcript seen on Northern blot may arise from alternative splicing of the SF 3' UTR, given the similarities in biology of SF and M-CSF, the nucleotide sequence and biologic significance of the 3.6 kb SF transcript species remains unknown.

Others have found constitutive expression of SF mRNA by human EC and bone marrow--derived reticulofibroblastic cells. In these studies, an inductive effect of IL-1β on SF mRNA concentration was reported. However, quantitative analysis of SF mRNA concentration was not performed, nor was SF protein measured.\textsuperscript{86} Using RT-PCR and Northern blot and RPA analyses, we were unable to confirm any effect of IL-1α on SF mRNA concentration in EC, DF, or LTBMC, nor could we measure any effect of IL-1α on the amount of membrane-bound SF protein expressed by EC, NFF, or DF. Because the sensitivity of our studies was such that less than a twofold change in SF mRNA concentration would have been detectable, and because the effect of IL-1α on mRNA levels of E-selectin and protein levels of cell-associated ICAM-1 and E-selectin were identical to previous reports,\textsuperscript{26,87,88} we conclude that IL-1α does not induce SF gene expression in the cells we tested. Nor is there evidence that IL-1α and IL-1β are different in this regard. We have previously found identical effects of IL-1α and IL-1β on EC and DF gene expression of GM-CSF, G-CSF, IL-1β, IL-6, and E-selectin. Additionally, in a limited series of experiments, treatment of EC with IL-1β had no effect on the concentration of SF mRNA (data not shown).

In summary, EC and DF that do not support hematopoi-
Fig 9. Effect of IL-1α on stromal cell membrane-bound SF, E-selectin, and ICAM-1. EC and NFF were treated with IL-1α (2.5 ng/mL) for 0, 4, or 24 hours. Membrane-bound SF was detected by indirect immunofluorescent staining using polyclonal rabbit anti-human SF (----) and FITC-conjugated goat antirabbit antibody. Normal rabbit serum and FITC-conjugated goat antirabbit antibody was used as a control (-----) for SF analysis. Membrane-bound ICAM-1 was detected by indirect immunofluorescent staining using RR1/1 MoAb (----) and FITC-conjugated goat antimouse antibody. Membrane-bound E-selectin (-----) was detected by indirect immunofluorescent staining using 1261.1 MoAb and FITC-conjugated goat anti-mouse antibody. 15-5-5's MoAb and FITC-conjugated goat anti-mouse antibody (----) were used as a control for ICAM-1 and E-selectin measurement.

Table 1: Effects of IL-1α on stromal cell membrane-bound SF, E-selectin, and ICAM-1.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Control</th>
<th>IL-1α 4h</th>
<th>IL-1α 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC SF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC ICAM-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC E-SELECTIN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFF SF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFF ICAM-1</td>
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</tbody>
</table>

et cell growth in long-term culture, as well as mixed stromal cells from LTBMC that do support progenitor growth, constitutively transcribe SF mRNA. IL-1α does not induce the accumulation of SF mRNA in any of these cell types. In addition, the ratio of exon 6α to exon 6 SF transcript is identical between these cell types and is not altered by exposure of stromal cells to IL-1α. Integral membrane SF is constitutively expressed by stromal cells and is not regulated by IL-1α. All three types of stromal cells studied expressed similar amounts of SF membrane-bound protein, although somewhat higher levels were found in skin-derived fibroblasts. Therefore, while the production of SF by stromal cells in LTBMC clearly contributes to the ability of these cells to support hematoipoiesis, the uniquely supportive function of LTBMC compared with EC or DF cannot be explained by differences in SF gene expression.

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