Vascular Cell Adhesion Molecule 1–Positive Reticular Cells Express Interleukin-7 and Stem Cell Factor in the Bone Marrow

By Phillip E. Funk, Robert P. Stephan, and Pamela L. Witte

In vitro studies have defined an essential role for stromal cells in supporting B-cell development, including production of lymphopoietic cytokines. It has been suggested that stromal cells are equivalent to adventitial reticular cells in the marrow; however, evidence of reticular cells producing cytokines has been difficult to obtain. Staining of bone marrow (BM) sections with antibodies to interleukin-7 (IL-7) showed a reticular pattern, mimicking that obtained using antibodies to vascular cell adhesion molecule 1 (VCAM-1), a molecule present on both stromal cells in vitro and reticular cells. To more closely examine cytokine production within normal marrow, an immunomagnetic separation scheme was devised to directly enrich VCAM-1+ stromal cells. Twenty to thirty percent of cells isolated in the VCAM-1+ fraction suggest that reticular cells are important to B-cell formation and may provide key regulatory elements.

Little is known about lymphohematopoietic cytokine production by marrow reticular cells. In particular, one cytokine, interleukin-7 (IL-7), is essential for normal B-cell development in vivo as well as in vitro, and expression of the IL-7 gene is limited to a few tissues and cell types.22–24 For example, only the fibroblastic-like stromal cells produce IL-7 in Whitlock-type LTBMCS. Two other cytokines are also singularly made by these stromal cells in Whitlock-type LTBMCS, stem cell factor (SCF) and macrophage colony-stimulating factor (M-CSF).22,24 Thus, expression of certain cytokines would be predicted in marrow reticular cells if they are (1) the functional counterpart of the cultured stromal cell and (2) necessary for B-cell formation. In the present study, we tested this hypothesis by developing a method to isolate and visualize reticular stromal cells directly from fresh BM cell suspensions using antibodies to VCAM-1. Our goal here was to study stromal cells immediately after isolation from the marrow to assess constitutive cytokine production in unperturbed marrow. These cells were characterized with respect to cell lineage markers and cytokine expression.

MATERIALS AND METHODS

Animals. Female BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN) and housed at the Animal Research Center, Maywood, IL.

Support by American Cancer Society Grants No. IM-612 and JFRA #409 (to P.L.W.) and a Loyola University Dissertation Fellowship (to P.E.F.). This work was performed in partial fulfillment of P.E.F.’s doctoral degree.

From the Departments of Cell Biology, Neurobiology and Anatomy and Microbiology/Immunology, Loyola University Medical Center, Maywood, IL.

Submitted December 22, 1994; accepted May 18, 1995.

Address reprint requests to Pamela L. Witte, PhD, Department of Cell Biology, Neurobiology and Anatomy, Loyola University, 2160 S First Ave, Maywood, IL 60153.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1995 by The American Society of Hematology.

Blood, Vol 86, No 7 (October 1), 1995: pp 2661-2671
Facility at Loyola University (Maywood, IL). Animals were 4 to 8 weeks old when killed.

**Immunohistochemistry of marrow frozen sections.** Tibial marrow plugs were gently forced from the marrow cavity with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), injected through a 25-gauge needle. Intact plugs were placed in O.C.T. compound (Miles Inc, Elkhart, IN) and snap frozen on dry ice. Frozen plugs were used the same day or stored overnight at −20°C. Eight-micron cross-sections were cut on an automated Kryostat (model no. 1720; Leitz, Rockleigh, NJ). Five individual sections were placed on each poly-l-lysine-coated microscope slide, fixed in cold (−20°C) acetone for 30 minutes, and air-dried. Each section was demarcated using a PAP pen (RP1, Mount Prospect, IL). The endogenous peroxidase activity was blocked with 0.3% H2O2 in methanol for 30 minutes at room temperature. The slides were washed three times in phosphate-buffered saline (PBS). The sections were then incubated for 30 minutes with 50% donkey or mouse serum to block nonspecific antibody binding. The sections were then incubated at room temperature for 60 minutes with 30 μL of either polyclonal rabbit antihuman IL-7 antibody (10 μg/mL; affinity purified; cross-reactive with mouse IL-7; Biosource International, Camarillo, CA) or rat-antimouse VCAM-1 monoclonal antibody (MoAb) (M/K2, 7.5 μg/mL; protein G purified; American Type Culture Collection, Rockville, MD). After washing three times in PBS, the sections were stained for 30 minutes with biotinylated donkey-antirabbit IgG or mouse-antirat IgG (Fab'2, Jackson Immunoresearch, West Grove, PA). After three more PBS washes, the sections were incubated for 30 minutes with the ABC Elite kit (1:100, Vector, Burlingame, CA). Horseradish peroxidase enzyme activity was detected by immersing the sections in 0.45 mg/mL diaminobenzidine (DAB; Sigma, St Louis, MO) for 15 minutes in buffer containing 0.15 NaCl, 0.05 mol/L TRIS, and 0.07% H2O2. The sections were counterstained with hematoxylin, washed, dehydrated in a graded ethanol (ETOH) series followed by xylene, mounted, and observed by bright field microscopy. Best results were obtained when the entire procedure was performed within 72 hours after procuring the marrow. Reaction and specificity of the rabbit-antihuman IL-7 for recombinant mouse IL-7 was tested in our lab by enzyme-linked immunosorbent assay (ELISA); no cross-reactivity with other cytokines was observed (data not shown).

**Reverse-transcription polymerase chain reaction (RT-PCR).** This was performed exactly as described in detail previously.11 RNA was isolated from unfractioned BM cell suspensions. Positive control RNA was prepared from the marrow stromal cells of Dr. P. Kincade, induced with lipopolysaccharide and cycloheximide. A 466 bp fragment of osteocalcin was detected from the marrow stromal cells in 0.45 mg/mL diaminobenzidine (DAB; Sigma, St Louis, MO) for 15 minutes in buffer containing 0.15 NaCl, 0.05 mol/L TRIS, and 0.07% H2O2. The sections were counterstained with hematoxylin, washed, dehydrated in a graded ethanol (ETOH) series followed by xylene, mounted, and observed by bright field microscopy. Best results were obtained when the entire procedure was performed within 72 hours after procuring the marrow. Reaction and specificity of the rabbit-antihuman IL-7 for recombinant mouse IL-7 was tested in our lab by enzyme-linked immunosorbent assay (ELISA); no cross-reactivity with other cytokines was observed (data not shown).

**Isolation of BM aggregates and cell preparation.** Mice were killed by cervical dislocation and the femurs removed. Marrow was flushed from the femurs with cold RPMI 1640 medium supplemented with 10% heat-inactivated FBS by inserting a 25-gauge needle attached to a 3-mL syringe. This cell suspension was then centrifuged at 1,000 rpm for 10 minutes, and the cells were resuspended by pipetting 10 to 15 times through a 5- or 10-mL glass serologic pipet. For aggregate isolation, cells were resuspended at 3 femur equivalents/mL after centrifugation and vigorously pipetted through a 5- or 10-mL pipet. One milliliter of this suspension was carefully layered over 3 mL of cold FBS in a 5-mL tube. Aggregates were allowed to settle for 15 minutes by placing the tube on ice in a vertical position. After 15 minutes, the lowermost 1 mL, which contained aggregates estimated to be 20 to 500 cells per aggregate, was collected with a Pasteur pipet.

A cocktail of 0.2% collagenase (grade CLS 3, lot no. FOD 671; Worthington, Freehold, NJ), 0.1% hyaluronidase (grade HSE, Worthington) and DNase I (Worthington) in RPMI 1640 + 10% FBS was used to digest aggregates into single cells. Cells were digested in this cocktail for 1.5 to 2 hours at 37°C with 70 rpm agitation on an orbital shaker. After digestion, cells were washed twice in cold RPMI 1640 with 10% FBS before subsequent manipulations.

**Immunomagnetic separation of VCAM-1+ cells.** Goat-antirat IgG (Fc-specific) magnetic beads (Advanced Magnetics, Cambridge, MA) were incubated for 15 minutes at 4°C 10 μg/mL rat IgG (chromatographically purified; Sigma) or a 1.500 dilution of ascites from SCID that had received M/K2 hybridoma cells (anti/VCAM-1)17 in a total volume of 4 mL. The cells were then washed three times with RPMI 1640 supplemented with 10% heat-inactivated FBS. A bead cell ratio of 40:1 was chosen by titration experiments based on the bead:cell ratio used to isolate endothelial cells.27 Cells from dispersed aggregates were incubated for 20 minutes at 4°C with rat IgG–coated beads in a total volume of 4 mL. Cells that bound rat IgG nonspecifically were then removed using a Biomag Separator (Advanced Magnetics). Negatively selected cells from this step were washed and then incubated for 20 minutes with MoAb M/K2–coated beads in a total volume of 4 mL. Cells were then subjected to another round of magnetic separation. Immediately after separation and without washing, approximately 1 × 107 positively selected cells in 100 to 200 μL were carefully pipetted onto Superfrost glass microscope slides (Fisher, Pittsburgh, PA) coated with 10 μg of CelTak (Collaborative Biomedical Products, Bedford, MA)26 and incubated at 37°C in a humidified incubator for 1 hour. The slides were then centrifuged at 350 rpm in microplate carriers in a Beckman GPR centrifuge with rotor GH3.7 (=30g) and subsequently stained for alkaline phosphatase (Kit 86C, Sigma), acid phosphatase (kit 386-A), naphthyl esterase (kit 91-A), chloroacetate esterase (kit 91-C), or acetyl cholinesterase activities or with one of several antibodies named below.

**Detection of osteocalcin protein by immunoperoxidase reaction.** Selected BM cells adhered to CelTak-coated slides were fixed and stained with goat-antimouse osteocalcin antiserum (Biomedical Research Incorporator, Stoughton, MA) as described below. The optimal concentration of antiosteocalcin (1:375 dilution, 4-hour incubation) staining was determined by titration on preosteoblast MC-3T3-E1 cells induced to express osteocalcin28 (gift of Dr Janet Stein, University of Massachusetts Medical Center, Worcester, MA). To induce E1 cells expression, E1 cells were plated on tissue culture dishes with 25 μg/mL ascorbic acid and 10 mmol/L β-glycerol phosphate (Sigma) according to the method of Quarles et al.11 Medium was replaced once a week and cells were used 23 days after being placed under inducing conditions. The secondary antibody was biotinylated donkey-antigoat IgG [Fab’2]; Jackson Immunoresearch.

**Uptake of acetylated low-density lipoprotein (acLDL).** To label normal marrow cells, 5 μg/mL of Dil-acLDL (Biomedical Technologies, Cambridge, MA) was added to the collagenase digestion cocktail at the beginning of incubation. As control, adherent cells from Whitlock LTBMCSs were incubated in an identical cocktail of collagenase and acLDL. The macrophages, but not the stromal cells in these cultures will take up acLDL.14 The LTBMCSs were then released using EDTA and silicon rubber policeman, washed in RPMI 1640 with 10% heat inactivated FBS, placed on CelTak coated slides, and treated identically to the VCAM-1–selected cells. The presence of red fluorescence within VCAM-1–selected stromal cells was judged by epifluorescence and phase microscopy.

**Immunofluorescence localization of alpha-smooth muscle actin.** Cells on CelTak–coated slides were fixed cold (−20°C) 95% ethanol and 5% acetic acid. The slides were then washed three times in cold PBS. After the washes, the cells were stained for 30 minutes with...
ISOLATION OF MARROW RETICULAR STROMAL CELLS

Fig 1. Immunoperoxidase reaction of antibodies to VCAM-1 and IL-7 on cryosections of BM. (A) rat anti-VCAM-1 MoAb; (B) control rat IgG; (C) rabbit anti-IL-7 affinity-purified antibody; (D) control rabbit IgG. Long arrows indicate the extravascular reticular pattern of deposition associated with reticular cells. Short arrows mark a VCAM-1+ endothelial cell lining a sinusoid (original magnification × 630).

80 μL per slide of MoAb 1A4 (mouse anti-a-actin IgG, ascites, 1:300 dilution, Sigma) in a humid chamber. Slides were washed again in PBS and then stained with fluorescein isothiocyanate-conjugated goat-antimouse IgG [F(ab')2] for 30 minutes. The slides were washed three times in PBS, mounted in phosphate-buffered glycerol, and observed by epifluorescence and phase microscopy. Primary cultured stromal cells from Whitlock LTBMCs, which express α-actin, were used as positive control cells.

Immunocytochemical staining for intracellular cytokine proteins. Cells on CelTak-coated slides were immediately fixed or held over-
night in tubes with desiccant before staining. Cells were fixed for 15 minutes in cold 95% ethanol with 5% acetic acid. The slides were then washed three times in PBS. Endogenous peroxidase activity was blocked with 0.3% H$_2$O$_2$ in methanol for 30 minutes at room temperature and the slides were again washed three times in PBS. At this point the cells were incubated for 1 hour with 50% donkey serum to block nonspecific binding of subsequent antibodies. Rabbit-anti-human IL-7 IgG (10 µg/mL; Biosource International) was applied using 80 µL per slide for 1 hour. Appropriate concentration was determined on IL-7 expressing stromal cells from LTBMCs as previously published. Slides were washed three times in PBS and stained with biotinylated donkey-antirabbit IgG [F(ab')$_2$]. After three more PBS washes, slides were stained with the ABC Elite kit (1:100; Vector). Horseradish peroxidase enzyme activity was detected by immersing the slides in DAB solution for 15 minutes as above. Slides were counter stained in hematoxylin. Cells to be stained for SCF or M-CSF proteins were fixed and washed as above. The cells were then incubated overnight with 80 µL/slide of rabbit-antimouse SCF antibody (5 µg/mL, protein A purified, Genzyme, Cambridge, MA) or for 1 hour with goat-antimouse M-CSF serum (1:200 dilution, kindly provided by Dr. E.R. Stanley, Albert Einstein College of Medicine, Bronx, NY). Both of these antibodies were previously titered by staining adherent cells from Whitlock LTBMCs. The specificity of antibody against SCF was verified by ELISA in our lab (data not shown). After washing and staining with appropriate secondary reagents as above, antibody binding was visualized using an alkaline phosphatase ABC kit with substrate kit (Vector) in the presence of 1 µl levamisole (Vector)/80 µL to block endogenous alkaline phosphatase activity. Slides were counterstained with neutral red. The cells were observed and scored by both bright field and phase microscopy.

RESULTS

Detection of reticular stromal cells and cytokines in BM sections. Immunoperoxidase staining of BM sections with antibodies against VCAM-1 (M/K2) showed a reticular pattern scattered throughout the section (Fig 1A, long arrows). Some sinusoidal endothelial cells were positive as well (short arrows). These observations confirmed previous reports that showed by light and electron microscopy that VCAM-1 is expressed primarily by adventitial reticular cells in marrow.'5-19 The distinctive extravascular reticular pattern is caused by the deposition of stain along the many long processes of adventitial reticular cells.'7,19 and is also observed by reactivity with alkaline phosphatase substrates.'9 This serves as comparison with the pattern observed with antibodies to IL-7 (Fig 1C). When localized in fresh marrow sections, the staining pattern of anti-IL-7 antibody was again reticular, similar to that of M/K2 except that staining was never found associated with endothelium. The deposit was clearly between, rather than within, the cytoplasm of hematopoietic cells and was found in cluster-like formations, evenly scattered throughout the marrow cross-sections. The pattern suggested that IL-7 protein was associated with adventitial reticular cells.

Because it is possible that the extracellular matrix could bind and localize IL-7 secreted outside the marrow, we wanted to confirm that IL-7 and other cytokines associated with stromal cells in primary Whitlock cultures SCF and M-CSF were produced within the marrow microenvironment. Therefore, RT-PCR was performed on total BM RNA. Transcripts for all three cytokine genes were detected (Fig 2).

Although these experiments suggested that the reticular stromal cells were possibly producing IL-7 in the BM, dual localization of cytokine protein and stromal cell markers has been difficult to achieve in tissue section because the stromal cell processes can only be clearly visualized by electron microscopy.'4,18,21 As an alternative approach, a method was developed to isolate the stromal cells so that associated cytokine products could be identified.

Conditions necessary to visualize stromal cells from fresh BM suspensions. We and others have been able to identify stromal cells from BM but only after at least 48 hours in culture. For this purpose, we used an enriched source of marrow stromal cells, cellular aggregates from femoral BM that contain up to five times the number of culturable stromal cells found in unfractioned marrow.'6 The aggregates were gently dispersed with a cocktail of selected collagenase, hyaluronidase, and DNase. After low-speed centrifugation of the cells onto CelTak-coated slides, large, alkaline phosphatase–positive cells were seen (Fig 3A). These were rare cells because only two to three per slide were observed, and similar cells could not be found on conventional cytocentrifuge preparations. The alkaline phosphatase–positive cells possessed several characteristics consistent with criteria used to define stromal cells in vitro: (1) large size; (2) a single, large, oval nucleus; (3) an abundant cytoplasm that was devoid of vacuoles or granules visible at the light microscopic level; and (4) hematopoietic cells often associated with their surfaces. These cells were tentatively termed reticular stromal cells and could be readily identified on the basis of their distinctive morphology alone.

Fig 2. RT-PCR to detect RNA transcripts for IL-7, SCF, and M-CSF in young adult mouse BM. The amplified products are depicted after ethidium bromide staining. The stromal cell line BMS2, which produces these cytokines, was a positive control. Negative controls included samples containing the RNA without reverse transcription (shown) and samples without RNA or cDNA (not shown). Bands: IL-7, 392 bp; SCF, 344 bp; M-CSF, 503 bp. Data shown represents one of two experiments using marrow pooled from two 5-week-old mice per experiment.
ISOLATION OF MARROW RETICULAR STROMAL CELLS

AcLDL Endothelial cells, macrophages
α-Actin Smooth muscle, pericytes, osteoclasts
Osteocalcin Osteoblasts
Acetylcholine Esterase Megakaryocytes
Chloroacetate Esterase Granulocytes

Alkaline Phosphatase Osteoblasts, reticular cells, granulocytes

Table 1. Percent Positive of Each Phenotype of Stromal Cells in the VCAM-1+ Fraction

<table>
<thead>
<tr>
<th>Exp</th>
<th>Alk Phos</th>
<th>Acid Phos</th>
<th>Naph Estr</th>
<th>Chloro Estr</th>
<th>AchE</th>
<th>α-Actin*</th>
<th>OC1</th>
<th>acLDLt</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>86</td>
<td>0</td>
<td>57%</td>
<td>0</td>
<td>94</td>
<td>1</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>98</td>
<td>0</td>
<td>43</td>
<td>2</td>
<td>0</td>
<td>78</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>96</td>
<td>ND</td>
<td>41</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>72</td>
<td>ND</td>
</tr>
</tbody>
</table>

All values are the average of at least two slides. One hundred cells with stromal morphology were scored per slide, except where noted.

Abbreviations: Alk phos, alkaline phosphatase; Naph estr, naphthyl esterase; chloro estr, chloroacetate esterase; AchE, acetylcholine esterase; OC, osteocalcin; ND, not determined.

* Detected by indirect immunofluorescence; 50 stromal cells were scored per slide.
† Uptake of Dil-labeled acLDL detected by fluorescence microscopy; 50 stromal cells were scored per slide.
‡ Intensity level of naphthyl esterase stain on stromal cells was always lower than that on BM monocytes.

Because they were too rare for quantitative analysis, the stromal cells were further enriched on the basis of VCAM-1 expression. The utility of this approach was justified in initial experiments using panning. We observed that virtually all culturable stromal cells (in 48 hours) were found in the VCAM-1+ selected fraction (data not shown). However, procuring the cells from the panning plates led to shearing of the cells. Further experiments using the fluorescence-activated cell sorting (FACS) (even macro sorting) were unsuccessful in enriching stromal cells, probably because the cells were fragile and destroyed by turbulence in the soft stream. Immunomagnetic bead separation was finally tried as a less traumatic means to enrich stromal cells.

Immunomagnetic separation of VCAM-1+ cells from collagenase dispersed aggregates yielded enrichment of cells morphologically similar to the rare cells seen previously (Fig 3B). VCAM-1+ cells with stromal morphology were always surrounded by many magnetic beads, often being partially obscured by them, which suggested that these cells indeed expressed the VCAM-1 molecule. Hematopoietic cells were also seen on these slides, but were rarely in close contact with magnetic beads, suggesting that they were VCAM-1- contaminants. Over the course of these experiments, an average of 9.4% ± 4.0% of all nucleated BM cells separated into the aggregate fraction, which is similar to our previous reports.16,22 In data collected from 19 experiments performed over 9 months, 7.0% ± 3.2% of marrow aggregate cells separated into the VCAM-1+ fraction and 64.2% ± 20.1% of aggregate cells into the VCAM-1- fraction. The total cell loss in individual experiments using magnetic bead selection varied from 0% to 50%, averaging 19% per experiment (in 12 of 19 trials, the loss was less than 19%). Approximately 23.7% ± 3.8% of the VCAM-1+ cells had the morphologic features used to identify stromal cells (above) and almost all of these reacted with alkaline phosphatase substrate (Table 1). It is possible that the total cell loss may impinge on the ultimate recovery of stromal cells; however, no cells with stromal cell morphology were seen on identically prepared slides with VCAM-1- cells, suggesting that all recoverable stromal cells were selected into the VCAM-1+ fraction. Alkaline phosphatase activity in the VCAM-1- fraction was limited to the neutrophilic population. Immunohistochemical staining of the VCAM-1+-selected stromal cells showed that they expressed the VCAM-1 molecule (data not shown). Therefore, we conclude that all putative reticular stromal cells express VCAM-1.

Relationship of VCAM-1+ stromal cells to other cell types. Several mesenchymal cell types could potentially be confused with reticular stromal cells, including osteoblasts, osteoclasts, endothelial cells, and smooth muscle cells. Also, megakaryocytes and resident macrophages are large cells that could potentially be misidentified as stromal cells when freshly isolated. Therefore, histochemical assays and antibody staining experiments were performed to characterize the stromal cells regarding features known to mark these other cell types (Table 2).

It has been proposed that stromal cells in hematopoietic LTBMCS are actually endothelial cells.38 Although several observations have argued against an endothelial origin for stromal cells in Whitlock-type LTBMCS, sinusoidal

Table 2. Markers Used to Discriminate Mesenchymal and Hematopoietic Lineages

<table>
<thead>
<tr>
<th>Marker</th>
<th>Expected Positive Cellular Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline Phosphatase</td>
<td>Osteoblasts, reticular cells, granulocytes (weak)</td>
</tr>
<tr>
<td>Acid Phosphatase</td>
<td>Neutrophils, osteoclasts</td>
</tr>
<tr>
<td>Naphthyl Esterase</td>
<td>Monocytes</td>
</tr>
<tr>
<td>Chloroacetate Esterase</td>
<td>Granulocytes</td>
</tr>
<tr>
<td>Acetylcholine Esterase</td>
<td>Megakaryocytes</td>
</tr>
<tr>
<td>α-Actin</td>
<td>Smooth muscle, pericytes, myofibroblasts, reticular cells</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>Osteoblasts</td>
</tr>
<tr>
<td>acLDL</td>
<td>Endothelial cells, macrophages</td>
</tr>
</tbody>
</table>

Table 3. Percent Positive Cytokine Proteins Detected in VCAM-1+ Stromal Cells

<table>
<thead>
<tr>
<th>Exp</th>
<th>IL-7*</th>
<th>Stem Cell Factor</th>
<th>M-CSF†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>83.0 ± 3.6</td>
<td>57.0 ± 6.2</td>
<td>5.0 ± 2.0</td>
</tr>
<tr>
<td>2</td>
<td>84.3 ± 10.7</td>
<td>61.3 ± 4.9</td>
<td>6.3 ± 2.1</td>
</tr>
<tr>
<td>3</td>
<td>84.0 ± 1.0</td>
<td>53.0 ± 1.7</td>
<td>6.3 ± 2.1</td>
</tr>
</tbody>
</table>

All values are the average of three slides ± SD. One hundred stromal cells were scored per slide, 1000 stromal cells per experiment. Optimum antibody concentrations were assessed in prior experiments using stromal cells from LTBMCS. Irrelevant rabbit IgG or goat serum controls gave negative or negligible staining in comparison with anticytokine antibodies. * Detected by immunocytochemistry with peroxidase visualization. † Detected by immunocytochemistry with alkaline phosphatase visualization.
endothelial cells were a likely component of the isolated VCAM-1+ cells. Endothelial cells (and macrophages) were marked by uptake of DiI-labeled acLDL during collagenase digestion. Collagenase did not affect the uptake of acLDL by macrophages from LTBMCS prepared in parallel as a control. VCAM-1+ cells were isolated and placed on slides; cells with stromal morphology were analyzed for positive fluorescence. Less than 15% of the stromal cells took up acLDL (Table 1). Other acLDL+ cells present in the VCAM-1+ fraction could be discerned from the stromal cells by phase-contrast microscopy as having a denser, more rounded nucleus, a granular cytoplasm, and higher ratio of nucleus to cytoplasm. Therefore, although endothelial cells appeared to be present in the VCAM-1+ fraction, they could be morphologically distinguished from stromal cells and did not interfere with subsequent studies. Contamination with macrophages was proven unlikely because the low level of naphthyl esterase activity of the putative reticular cells was clearly much less than that observed in marrow monocytes and macrophages found on the same slides or slides of unfractoned marrow (Table 1).

It has also been suggested that marrow stromal cells may be related to osteoblasts.39 Osteocalcin is a bone matrix protein produced exclusively by cells in the later stages of osteoblast differentiation.40,41 VCAM-1+ stromal cells exhibited undetectable levels of this protein, shown by immunoperoxidase reaction with specific antibodies (Table 1). Therefore, VCAM-1+ isolated stromal cells did not have characteristics of osteoblasts or their immediate precursors.

All cells identified morphologically as stromal cells were negative for acid phosphatase, chloroacetate esterase, and acetylcholine esterase (Tables 1 and 2). Also, the regular shape of the nucleus and clear cytoplasm of the stromal cells differed from those of monocytes/macrophages, megakaryocytes, and osteoclasts. Notably, some megakaryocytes appeared among the VCAM-1+ cells, always in contact with antibody-coated beads. Identification of megakaryocytes was confirmed by acetylcholine esterase staining, which completely distinguished them from the stromal cells. In addition, the stromal cells were invariably uninnucleate and acid phosphatase negative, distinguishing them from osteoclasts.42 Lack of chloroacetate esterase activity distinguished them from the granuocyte lineage (Table 1).

Finally, stromal cells in culture express α-actin3,8,32-34 (and our observations). Notably, the majority of the freshly isolated stromal cells studied here also possessed α-actin filaments (Table 1) as determined by indirect immunofluorescence staining using a MoAb against α-actin. This finding, in particular, strengthened the comparison between the putative reticular cells isolated in the VCAM-1+ fraction and stromal cells in culture.

Detection of intracytoplasmic cytokine proteins in VCAM-1+ stromal cells. Stromal cells are essential for the formation of B-lymphocytes in vitro and produce cytokines important for B-cell development, particularly IL-7 and SCF.9,13 We reported earlier that mRNA for IL-7, SCF, and M-CSF can only be detected in the stromal cells in Whitlock-type cultures, in which B-lineage proliferation and differentiation take place. If the candidate stromal cells identified in the VCAM-1+ fraction provide similar lymphocyte support functions in vivo, a prediction would be that at least some of the cells would express IL-7 and/or SCF proteins. In addition, it was anticipated that most stromal cells would make M-CSF as almost all hematopoietic-supporting stromal cell lines and primary stromal cells constitutively produce abundant M-CSF.8,13 Specific antibodies against IL-7, SCF, and M-CSF were used to detect the proteins by immunocytochemistry. All three cytokines were easily detected within the cytoplasms of most primary cultured stromal cells from Whitlock-type LTBMCS (and data not shown). Three independent experiments using marrow from 4- to 8-week-old mice were performed for each cytokine (Table 3 and Fig 4). A majority (>80%) of anti–VCAM-1+–isolated stromal cells expressed IL-7 protein. Notably, no IL-7 staining was observed among the VCAM-1+ cells. Approximately half of the stromal cells stained positively for SCF, and less than 7% positive stromal cells were detectable with antibodies to M-CSF. Therefore, at least one cytokine, IL-7, that has been predicted to be pivotal to B lymphopoiesis in vivo is a product of normal BM reticular cells. One third to half of this population also expressed detectable SCF, but the variability of detectable SCF production predicts that marrow stromal cells may be a functionally heterogeneous cell population.

DISCUSSION

Several activities that impinge on the regulation of lymphohematopoiesis have been ascribed to stromal cells in long-term BM cultures. Among the best defined of these activities are those that are important early in B-lineage formation. Stromal cells in the adherent layer produce growth and differentiation factors that allow B lymphopoiesis to persist in culture, including IL-7 and SCF.6 Lymphoid precursors interact with highly spread adherent stromal cells in LTBMCS,5,8,13 and VCAM-1 is a stromal-associated adhesion molecule that participates in this interaction.37,43-46 A similar interaction is seen with reticular stromal cells in the BM,18,39 which can be abolished by injection of MoAb (MK2) to VCAM-1.32 These observations have greatly contributed to the notion that reticular stromal cells are fundamental players in the regulation of B lymphopoiesis and many other aspects of hematopoiesis. However, recent studies suggest that the stromal cells found in LTBMCS may be
derived from rapidly proliferating mesenchymal precursors and not the reticulum cells present in the marrow parenchyma.\textsuperscript{6,7} If this is correct, conclusions about the lymphopoietic activity of marrow reticular cells extrapolated from culture data may not be entirely justified. Here, we tested the hypothesis that marrow reticular stromal cells perform lymphopoietic support functions in vivo, as do cultured stromal cells, by producing the necessary cytokines. Our results strongly suggested that a majority of freshly isolated stromal cells constitutively produce one or more growth factors involved in B lymphopoiesis.

A method was first devised to isolate and greatly enrich marrow stromal cells, and this was successfully accomplished with the use of magnetic beads labeled with antibody to VCAM-1. Evidence that the cells studied here were reticular stromal cells of the BM was based on the patterns of tissue expression seen with stromal cell proteins. Reticular cells observed in histologic preparations express alkaline phosphatase and VCAM-1, as do the isolated cells studied here.\textsuperscript{1,7,10,35} The reticular-like pattern of immunoperoxidase deposition with antibody to IL-7 provided additional compelling evidence.

Quantitative analysis of the number of recovered cells with stromal morphology and alkaline phosphatase activity leads to an estimate of 1.2 to 1.9 x 10\textsuperscript{6} stromal cells in aggregates per femur. Thus, stromal cells are 237 times more frequent in the femur than in collagenase-digested, unselected aggregates and about 700 times more common than in unfractoned marrow. The previous estimate of the frequency of stromal cells in aggregates was about 700 per femur, based on their ability to attach and spread into morphologically identifiable stromal cells by 48 hours of culture.\textsuperscript{16} This suggests that as few as 6% of stromal cells in the marrow survive in culture for 48 hours. Whether these surviving cells can proliferate to form all of the stromal cells found in LTBM C cultures or if LTBM C stromal cells indeed arise from a mesenchymal precursor needs to be resolved. In preliminary experiments, we have had some success in culturing the VCAM-1\textsuperscript{+} stromal cells isolated by the Mini-Mac magnetic bead system. In these experiments, both proliferative and nonproliferative, alkaline phosphatase-positive stromal cells have been noted (R.P.S., unpublished observations, December 1994). The present results suggest that if most LTBM C stromal cells do arise from a mesenchymal precursor, differentiation to a phenotype similar to the reticular stromal cell occurs within a short time in culture.

We conclude that most stromal cells constitutively express VCAM-1 in young adult marrow, based on the lack of cells with stromal morphology and greatly diminished numbers of culturable stromal cells (by 6 days) in the VCAM-1\textsuperscript{-} fraction. This is similar to primary stromal cells in lymphopoietic cultures; however, the cultured cells show considerable heterogeneity in the relative amount of VCAM-1 expressed on the cell surface.\textsuperscript{8} Whether presentation of VCAM-1 by the isolated stromal cells is also heterogeneous is unknown and this could be important to stromal cell function in vivo.

As yet there is no specific marker for BM reticular stromal cells, so conclusive demonstration that these are reticular cells is not yet feasible. However, the experiments herein either eliminate or render unlikely several alternate cell types that might be confused with stromal cells once the marrow is dispersed, including macrophages, megakaryocytes, osteoblasts, osteoclasts, endothelial cells, and smooth muscle cells. The uncultured stromal cells studied here were found to possess smooth muscle \(\alpha\)-actin filaments, as do stromal cells in vitro.\textsuperscript{72-74} The parallel with smooth muscle cells has been explored by Galmiche et al,\textsuperscript{7} who recently reported that stromal cells in culture express other markers of smooth muscle cells. It is interesting that the stromal cells originated from \(\alpha\)-actin-negative precursors in culture, making it possible that \(\alpha\)-actin expression is induced by culture conditions. Smooth muscle cells are rare in marrow tissue sections, appearing as a single cell lining around the infrequent arterial vessels, and they do not extend processes among the hematopoietic cells.\textsuperscript{47} Recently, VCAM-1 expression has been reported by coronary artery and aortic smooth muscle cells during graft rejection or induction with cytokines.\textsuperscript{36,69} However, we have found little evidence for constitutive expression of VCAM-1 by smooth muscle cells that surround arterioles in the BM, even when some endothelial cells in the same general location had detectable stain (P.W., unpublished observations, August 1994). Notably, different cytokines stimulate VCAM-1 expression by endothelial and smooth muscle cells.\textsuperscript{86} Further studies using other markers of smooth muscle cells would be required to absolutely determine if the isolated VCAM-1\textsuperscript{-} stromal cells are smooth muscle in origin. Another cell type known to express \(\alpha\)-actin is the pericyte, a cell found lining the abluminal side of blood vessels,\textsuperscript{60,101} as do adventitial reticular cells. Moreover, pericytes proliferate readily in culture. However, the existence of pericytes in the marrow is controversial.\textsuperscript{54,55} Nevertheless, expression of \(\alpha\)-actin makes it possible that the VCAM-1\textsuperscript{-} stromal cells in vivo are smooth muscle-like, contractile cells with the ability to actively sort hematopoietic cells as adventitial reticular cells are proposed to do.\textsuperscript{19} Expression of VCAM-1, commonly seen on activated endothelial cells, and \(\alpha\)-actin, a smooth muscle characteristic, makes it possible that the VCAM-1\textsuperscript{-} stromal cells represent a uniquely differentiated cell type, which possesses a phenotype intermediate to endothelial and smooth muscle cells. Further investigation with more definitive lineage markers and the discovery of a stromal cell-specific marker would help resolve this possibility.

Intracytoplasmic IL-7 and SCF proteins were detected in the stromal cells from fresh marrow. In particular, the expression of IL-7, which appeared confined to the reticular stromal compartment, argues that the VCAM-1\textsuperscript{-} stromal cell explored in this report is likely a differentiated mesenchymal cell that provides a unique function in the marrow. IL-7 appeared to be expressed by nearly all freshly isolated stromal cells, similar to the pattern seen with stromal cells isolated from Whitlock-type cultures. Perhaps this is surprising in light of the fact that B-lineage cells comprise only about 25% of adult marrow cells and IL-7-responsive B-cell precursors are a small subset. One explanation may be the age of the animals analyzed, which is optimal for securing lymphopoiesis-supporting stromal cells in Whitlock-type LTBM C. The elevated levels of lymphopoiesis in 1- to 2-month-old mice\textsuperscript{64,66} may be a result of widespread expression.
of IL-7 by reticular stromal cells. However, we do not yet know how many of the stromal cells are transcriptionally active and cannot by the present analysis eliminate the possibility that protein detected in the cytoplasm was taken up from an exogenous source. It will be interesting to resolve whether IL-7 production by stromal cells is modified in situations of decreased lymphopoiesis, such as aging, pregnancy, and perturbations of marrow homeostasis.\(^\text{14,57}\)

SCF has broad hematopoietic effects\(^\text{9,65}\) but was only found in about half of the stromal cells. Our results suggest one of two possibilities: (1) production of this cytokine may discriminate two distinct types of stromal cells or (2) stromal cells may profoundly regulate the amount of SCF produced. The potent effects of SCF as a synergistic factor in combination with other hematopoietic cytokines may mean that widespread expression of SCF is not essential. Rather, small, focal amounts may be more effective. One prediction would be that stromal-associated SCF will be found in specific marrow regions such as the subendosteal area where proliferation of stem cells is greatest.\(^\text{9,66}\) Immunocytochemistry of SCF protein in frozen marrow sections has been less definitive than that of IL-7 and may require resolution at the level of electron microscopy (data not shown).

A particularly revealing comparison between the uncultured stromal cells and cultured stromal cells was the expression of M-CSF. M-CSF is expressed constitutively by primary cultured stromal cells and by most stromal cell lines.\(^\text{5,13,43,46}\) Based on this data, it has been suggested that M-CSF production by stromal cells is important in vivo, possibly to maintain resident marrow macrophages, and osteoclast function is compromised in mice with mutations in the M-CSF gene.\(^\text{66}\) However, very few freshly isolated reticular stromal cells possessed detectable M-CSF protein, suggesting that the normal source of M-CSF is likely to be from another cell type. Our data also imply that M-CSF expression by cultured stromal cells may be induced by culture conditions.

Our studies establish a baseline expression of three hematopoietic cytokines by marrow reticular stromal cells in young adult mice and suggest patterns of stromal cell heterogeneity heretofore unappreciated. Such a foundation is critical to understanding the importance of stromal cell functions during hematopoietic recovery after BM transplantation or treatment with irradiation or chemotherapeutic drugs.

**ACKNOWLEDGMENT**

We thank Muriel Hergott, Lisa Frantsve, and Michael Peters for providing technical assistance; Dr Phong Le for many helpful suggestions; Dr Virginia Sanders for critique of the manuscript; Drs Janet Stein and E.R. Stanley for gifts of cell lines or antibodies; and Dr Thomas Ellis and Patricia Simms of the Loyola University FACS Core Facility for generous help with flow cytometry and cell separation methodologies.

**REFERENCES**


53. Weibel ER: On pericytes, particularly their existence on lung capillaries. Microvasc Res 8:218, 1974


62. Zwebo KM, Wypych J, McNiece IK, Lu HS, Smith KA, Kar-


Vascular cell adhesion molecule 1-positive reticular cells express interleukin-7 and stem cell factor in the bone marrow

PE Funk, RP Stephan and PL Witte